Entomology in Focus 4

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Short Views on Insect Genomics and Proteomics

Insect Proteomics, Vol. 2

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Short Views on Insect Genomics and Proteomics

Insect Proteomics, Vol.2

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Short Views on Insect Genomics and Proteomics, Volumes 1 and 2

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Preface

Insects are the most successful group of animals on the planet and are ecologically and economically extremely important. Today's entomology field has gone beyond borders and is termed as a "super science." With its multidisciplinary approach, entomology explores new scientific frontiers. It has emerged to provide some of the most powerful tools in resolving fundamental biological questions and problems using genomic (genome sequencing, assigning functions to genes, determining genome architecture) and proteomic (nature of proteins, 3D structure, posttranscriptional modifications) approaches.

Whole-genome sequence projects for insect model organisms (29 insect species completed and many more under way) and the concurrent growth of sequence databases provide the biological sciences with invaluable sources of information. The two volumes of this book are intended to share the efforts of major contributors with the genomic and proteomic communities. This will pave the way toward the development of new and innovative approaches to improve public health and agriculture using effective and ecologically sound pest management systems.

We therefore decided to create an up-to-date reference that would provide a firm basis for understanding the past and current genomic and proteomic research conducted in entomology. To do this, we decided to bring together leading world scientists in molecular entomology and biotechnology to share their past experiences in the development of this field, to summarize the current state of the art, and to offer hypotheses and predictions to set a framework for future research. This book is composed of volumes 1 and 2 with 18 chapters.

Volume 1 *Short Views on Insect Genomics*

The first volume presents 8 chapters that address genomic approaches currently employed using various model organisms: body lice, whitefly, aphid, *Drosophila*, mosquitoes, lepidopterans, and others.

Chapter 1 provides a detailed story of the body louse genome project and its significance in understanding fundamental questions about the biology of lice and their endosymbionts. Of importance, it discusses the use of reverse genetics (RNAi) to answer questions about the role of specific genes in biological processes (Barry R. Pittendrigh, University of Illinois, USA).

Chapter 2 addresses advances in the genomics of the whitefly, *Bemisia tabaci*, an insect pest and plant virus vector. It also discusses the interaction between insects and viruses and the development of control strategies using RNAi approaches (Murad Ghanim, Agricultural Research Organization, Israel).

Chapter 3 provides an update on the scope and scale of the genomic data of Lepidoptera that is available in public databases and discusses the current status of lepidopteran genome projects. Special attention is drawn to (1) *Elongation factor-1α*, (2) *Wingless*, (3) *Cytochrome c oxidase I*, and (4) *ribosomal DNA and RNA* (América Nitxin Castañeda-Sortibrán, Universidad Nacional Autónoma de México).

Chapter 4 deals with the genetic and molecular mechanisms underlying the evolution of different aphid biotypes with respect to naturally occurring host plant resistance (Andy Michel, Ohio State University, USA).

Chapter 5 presents integrative genomic approaches used in studying epigenetic mechanisms of phenotypic plasticity in the pea aphid. It describes how epigenetic mechanisms (DNA methylation and chromatin remodeling) play an increasingly important role in winged vs wingless polyphenism in this highly adaptable species (Gael LeTrionnaire, INRA, France).

Chapter 6 provides an introduction to the concepts behind the dynamic and powerful field of insect regulatory genomics. It describes successful strategies and techniques for finding regulatory elements in model insect species like *Drosophila*, current efforts to extend them to evolutionarily diverged non-model organisms, and potential applications of this information using such approaches as gene transfer and RNAi (Marc S. Halfon, University at Buffalo-State University of New York, USA).

Chapter 7 presents a comprehensive coverage of comparative genomics of transcription factor binding in *Drosophila* by using ChIP-Chip, ChIP-Seq, and DamID techniques to discover a deeper understanding of genomic regulatory mechanisms (Steven Russell, University of Cambridge, UK).

Without the application of bioinformatics, the growth of genomic and proteomics would be limited. Chapter 8 focuses on a machine learning approach (ClanTox, NeuroPID, TOLIPs) to discover short bioactive proteins and peptides from insect genomes (Michal Linial, the Hebrew University of Jerusalem, Israel).

Volume 2 *Short Views on Insect Proteomics*

The second volume presents comprehensive and cutting-edge studies with emphasis on proteomics. It comprises ten chapters which constitute a key reference manual for everyone involved in insect biochemistry, molecular genetics, molecular

evolution, insect bioinformatics and structural biology, applications of insect biotechnology, insect "omics," and related fields.

Ticks transmit viral diseases to livestock, which are of great economic importance worldwide. Chapter 1 focuses on ticks (blood-sucking parasite) and recent developments in the field of sialomes (salivary gland proteomes). It discusses the regulation of host hemostasis and the molecular immune mechanisms behind it. It also discusses the utilization of salivary gland proteins in vaccines to control vector-borne diseases (Youmna M'ghirbi, University of Tunis El-Manar, Tunisia).

Current proteomic approaches rely on the application of mass spectrometry to protein molecules. Chapter 2 describes qualitative and quantitative proteomic methods for the analysis of the *Anopheles gambiae* mosquito proteome with emphasis on circadian changes in expression (G. E. Duffield, University of Notre Dame, USA).

Chapter 3 reviews recent advances in the knowledge of the lepidopteran digestive system. Key topics include the architecture, structure, and function of the lepidopteran peritrophic matrix (Dwayne D. Hegedus, Agriculture and Agri-Food Canada, Canada).

Many key agents protect insects from injury at low temperatures. Chapter 4 documents cold adaptation responses in insects and other arthropods using an "omics" approach (Duško P. Blagojević, University of Belgrade, Serbia).

Chapter 5 presents evidence for the evolutionary extinction of enzyme and molecular systems that engage and utilize the nonstandard amino acid, selenocysteine, in insects (Marco Mariotti, Centre de Regulació Genòmica, Barcelona, Spain).

Chapter 6 highlights recent progress in understanding the mechanisms behind the insect innate immune response with the silkworm, *Bombyx mori*, as a model organism. It reviews the characteristic features of antibacterial proteins and antimicrobial peptides (AMPs) produced by insects against pathogens, their modes of action, and current and potential medical applications of these molecules (Chandan Badapanda, Xcelris Genomic Research Center, India).

Chapter 7 takes the reader to the post genomic era where insects have become important models for applied sciences. This chapter describes the use of insect cell lines derived from model organisms like *Bombyx mori* as expression systems for vaccines and other peptides and proteins and the use of advanced protein expression systems based on the *B. mori* nucleopolyhedrovirus (BmNPV) bacmid (Enoch Y. Park, Shizuoka University, Japan).

Chapter 8 concentrates on the use of insects and their associated microorganisms as an important resource in diverse industries, especially for the production of industrial enzymes, microbial insecticides, and many other substances (Anthony Ejiofor, Tennessee State University, USA).

Chapter 9 deals with the special structure and properties of spider silks and their biotechnological applications (Daniela Matias de C. Bittencourt, Brazilian Agricultural Research Corporation, Brazil).

Chapter 10 focuses on the development, properties, and application of nanoparticles derived from plants producing bioactive compounds for use as novel agents to control human and insect pests (K. Murugan, Bharathiyar University, India).

It is our pleasure to launch the twin volumes of *Short Views on Insect Genomic and Proteomics*. The reader will find a wide variety of topics addressed in detail, which will help them update their knowledge of insect genomics and proteomics.

Manhattan, KS, USA — Chandrasekar Raman
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The chapters of this book series (Volumes 1 and 2) are organized to present many experts' contributions – highlighting their current lab research – to provide an overview of current and prominent advances in insect genomics and proteomics, and biotechnology, which will help students and researchers to broaden their knowledge and to gain an understanding of both the challenges and the opportunities behind each approach.

We collectively called the volumes *Short Views on Insect Genomics and Proteomics* with the support of Springer Science Media. I would like to extend my sincere gratitude to Prof. Cônsoli L. Fernando for approving this project. It was a really wonderful opportunity to work with the Springer editorial team (Dr. Zuzana Bemhart, Senior Publishing Editor; Mariska van der Stigchel, Editorial Assistant; Dr. William F. Curtis, Executive Vice President; Dr. Jacco Fllipsen, Vice President; Dr. Sadie Forrester, Executive Editor) and many more from the Springer family. We apologize to those whose work could not be cited owing to space considerations as well as any errors that may have occurred in the text, and we would be grateful to receive any comments or suggestions about improvements for further editions.

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form. I also thank my Prof. Gerald Reeck, Department of Biochemistry and Molecular Biophysics, Kansas State University, for providing encouragement and support throughout.

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Contents of Volume 1

Contents of Volume 2

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Chapter 1
Exploring the Sialomes of Ticks

Youmna M'ghirbi

Abstract Ticks (Acarina) are obligate blood-feeding arthopods that vector human and animal pathogens, causing typhus, Lyme disease, Rocky Mountain spotted fever, tick-borne relapsing fever, babesiosis, Q fever, arboviruses, anaplasmosis, and ehrlichiosis. Among the specializations required for this peculiar diet, tick saliva, a fluid once believed to be relevant only for lubrication of mouthparts and water balance, is now well known to be a cocktail of potent antihemostatic, anti-inflammatory, and immunomodulatory molecules that helps these arthropods obtain a blood meal from their vertebrate hosts. The repertoire of pharmacologically active components in this cocktail is impressive as well as the number of targets they specifically affect. These salivary components change the physiology of the host at the bite site, and, consequently, some pathogens transmitted by ticks take advantage of this change and become more infective. Tick salivary proteins have therefore become an attractive target to control tick-borne diseases. Recent advances in molecular biology, protein chemistry, and computational biology are accelerating the isolation, sequencing, and analysis of a large number of transcripts and proteins from the saliva of different ticks. Many of these newly isolated genes code for proteins with homology to known proteins allowing identification or prediction of their function. These and other molecules from genome and proteome sequences offer an exciting possibility to identify new vaccine antigens, potential biopharmaceuticals, antimicrobial peptides, and other novel human therapeutics.

Keywords Sialomes • Ticks • Acarina • Sialotranscriptome • Sialoproteome • Pharmacologically active components

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Abbreviations

ADP	Adenosine diphosphate
APTT	Activated partial thromboplastin time
ATP	Adenosine triphosphate
BIP	B-cell inhibitory proteins
BmAP	*Boophilus microplus* anticoagulant protein
BmTI-A	*Rhipicephalus microplus* trypsin inhibitor-A
BPTI–Kunitz	Basic protease inhibitor–Kunitz type
cAMP	Cyclic adenosine monophosphate
Dc	Dendritic cell
ECM	Extracellular matrix
ETC	Extrinsic tenase complex
FIXa	Factor IXa
FVIII	Factor VIII
FX	Factor X
FXa	Factor Xa
GP IIb–IIIa	Glycoprotein IIb–IIIa
IC_{50}	The concentration of an inhibitor where the response (or binding) is reduced by half
IFN	Interferon
IL	Interleukin
Ir-CPI	*Ixodes ricinus* contact phase inhibitor
IRS-2	*I. ricinus* serine proteinase inhibitor (serpin)
Isac	*I. scapularis* anticomplement
ISL929	*Ixodes scapularis* salivary proteins
MIF	Macrophage migration inhibitory factor
NCBI	National Center for Biotechnology Information
NK	Natural killer
OmCI	*Ornithodoros moubata* complement inhibitor
PGE2	Prostaglandin E2
PGF2a	Prostaglandin F2
PT	Prothrombin time
RaHBP	*Rhipicephalus appendiculatus* histamine-binding salivary protein
Salp	Salivary protein
SAT	Saliva-assisted transmission
SG	Salivary gland
SGE	Salivary gland extract
SHBP	serotonin- and histamine- binding protein
TAI	Tick adhesion inhibitor
TAP	Tick anticoagulant peptide
TdPI	Tick-derived protease inhibitor
TF	Tissue factor
tHRF	Tick histamine release factor
TT	Thrombin time

1.1 Introduction

Ticks are hematophagous ectoparasites of terrestrial vertebrates and are of great medical and veterinary importance, mainly because they are vectors of diseases affecting humans, livestock, and companion animals. Moreover, tick feeding can cause direct damage to their hosts such as significant blood loss as well as paralysis, toxicosis, irritation, and allergy [1]. Tick saliva contains a cocktail of potent pharmacologically active components able to disarm the host hemostatic system [2, 3] and alter the inflammatory and host immune responses [4, 5]. The molecules present in tick saliva range from lipids to large proteins and represent a plethora of biological activities (antihemostatic, anti-inflammatory, and immunomodulatory molecules). These molecules modify the physiology of their hosts at the tick-bite site, allowing these ectoparasites to obtain a blood meal from the host [3, 6–8]. In addition to its role in feeding and other functions related to ion and water handling, tick saliva may potentiate the transmission and establishment of tick-borne pathogens, and therefore, immune responses to tick saliva can confer protection against pathogen transmission [8–11]. Adaptation of ticks to their natural hosts has resulted in their ability to modulate the host immune and hemostatic response with their saliva. However, with non-natural hosts, tick feeding often results in immune and allergic responses, presumably to the injected salivary proteins, resulting in tick rejection [12]. Accordingly, the identification and characterization of tick salivary proteins may lead to the discovery of novel pharmacological agents, and it may help in the identification of potential vaccine candidates to control tick-borne diseases [8, 13].

Recently, salivary gland transcriptomic and proteomic analyses of several hard and soft ticks have been performed, providing data sets that are invaluable for a better understanding of tick sialomes and the immunobiology at the tick–host–pathogen interface [7, 14, 15]. Three main surprises arose with this approach:

1. The first was that the repertoire of tick salivary gland transcripts and proteins is much more broad and complex than anticipated, containing hundreds to thousands of different proteins, many of which are novel, since they produce no similarities to other proteins in the databases, such as the nonredundant database of the National Center for Biotechnology Information (NCBI). The authors of those studies classified the salivary transcripts and proteins they found as putative secreted or possible housekeeping groups and then into different groups according to their known or predicted biological function. Most such putative secreted proteins have unknown functions but, if secreted into their hosts, probably have antihemostatic, anti-inflammatory, immunomodulatory, or even anti-angiogenic or antimicrobial activity [15]. Regarding the probable housekeeping proteins identified, the authors suggested that their sequences may help to identify novel secreted protein families if identified in proteome experiments.
2. Another surprise was that the most abundant tick salivary proteins are members of multigene families. For some of these protein families, it is known that they are differentially expressed as feeding progresses; thus, at the last day of feeding, the tick is producing a different family member in saliva than that produced at the first day and may thus be evading the host immune response.

3. The third surprise, and challenge, was that we cannot anticipate at all the function of the majority of the tick salivary proteins. Indeed, for any tick species with a known transcriptome, less than 5 % of the proteins have been expressed and their function verified. Whole protein families await functional identification.

1.2 How Ticks Evade the Host

1.2.1 Tick Compounds Affecting Host Hemostasis

Hemostasis is an efficient mechanism that controls blood loss following vascular injury. Ticks attempting to obtain a blood meal face the vertebrate host's hemostatic system whose role is to prevent blood loss after tissue injury. The three branches of the hemostatic system, vasoconstriction (reduction of the blood flow), platelet aggregation (formation of the platelet plug), and the blood coagulation cascade (formation of the blood clot), pose a real threat to ticks when obtaining blood from the host. These three branches are well interconnected, making hemostasis a redundant system. The redundancy of the system is exemplified during platelet activation; these cells are essential in forming the platelet plug, but additionally, when activated, they release two potent vasoconstrictors, serotonin, and thromboxane A2, resulting in a decrease of blood flow. Additionally, activated platelets expose a negative charge on their membrane comprising exposed phosphatidyl serine phospholipids. These phospholipids are used for the formation of protein complexes such as the "tenase complex" composed of factor VIII (FVIII), factor IXa (FIXa), and factor X (FX), which is required for the formation of factor Xa (FXa) and the activation of the blood coagulation cascade. Another example of redundancy is in the blood coagulation branch. In addition to being a crucial enzyme in this cascade, thrombin cleaves the thrombin receptor on platelets, causing them to activate and aggregate. Therefore, the hemostatic system poses interesting problems for blood feeders, including specificity and redundancy [16].

How did ticks solve these problems? Ticks have been in the business of blood feeding for a long time. At least 120 Ma of evolution and adaptation to their host's hemostatic system have created a repertoire of potent bioactive salivary molecules with vasodilatory, antiplatelet, and anticoagulant activities [17, 18]. In many cases, molecules display more than one antihemostatic activity to combat the specificity and redundancy of the hemostatic system. Differences in the antihemostatic repertoires suggest that antihemostatic mechanisms in hard and soft ticks evolved independently [19]. Saliva of the same tick species can contain simultaneously more antihemostatic molecules, inhibiting different arms of the hemostatic system. However, it is important to note that the antihemostatic repertoire in ticks differs between species as well as across genera, and there is no tick species whose full antihemostatic capacities have been exhaustively explored and described [18]. Examples of the different molecules which have been characterized from the saliva of different ticks and examples of their potent biological activities will be described in this chapter (Table 1.1).

Table 1.1 Molecules characterized from the saliva of different tick species and their most potent biological activities

Tick species	Molecule	Molecular weight	Target and/or function	Nature of biochemical	References
Vasodilatation					
Ixodes scapularis	Prostacyclin ($PG1_2$)		Vasodilatation	Eicosanoïde	[20]
	tHRF	20 kDa	Vasodilatation	Ligand of histamine	[21]
Ixodes ricinus	IRS-2	38 kDa	Cathepsine G, chymase	Serpin	[22]
Amblyomma americanum	Prostaglandin E_2 and $F_2\alpha$	352.4 kDa	Vasodilatation	Prostaglandins	[23, 24]
Boophilus microplus	Prostaglandin E_2		Vasodilatation	Prostaglandin	[25]
Platelet aggregation inhibitors					
Argasidae	Apyrase		ATP, ADP	Apyrase	[26, 27]
Ornithodoros moubata	Moubatin	17 kDa	Collagen receptor	Lipocalin	[28]
	Disaggregin	60 kDa	Antagonist of integrin	Peptide	[29]
Ornithodoros savignyi	Savignygrin	14 kDa	Antagonist of integrin	Disintegrin	[30]
	Apyrase				[31]
	Savignin		Antithrombin		[32]
Ornithodoros moubata	Apyrase				[33]
	Disagregin				[29]
	Moubatin				[34]
	Tick adhesion inhibitor (TAI)				[35]
Ixodes scapularis	Apyrase	62 kDa	ATP, ADP	Enzyme	[36]
Ixodes scapularis, *Ixodes pacificus*	Ixodegrins	14 kDa	Antagonist of integrin	Peptide	[37]
Ixodes ricinus	IRS-2	42 kDa	Thrombin	Lipocalin	[22]
Haemaphysalis longicornis	Longicornin	16 kDa	Collagen receptor	Lipocalin	[38]
Dermacentor variabilis	Variabilin (GPIIa–IIIb antagonist)	5 kDa	Antagonist of integrin	Lipocalin	[39]
	Madanin 1 and 2				[40]

(continued)

Table 1.1 (continued)

Tick species	Molecule	Molecular weight	Target and/or function	Nature of biochemical	References
Amblyomma americanum	Americanin		Thrombin		[41]
Boophilus microplus	Antithrombin				[42]
Anticoagulation and fibrinolysis					
Ornithodoros moubata	TAP	7 kDa	FXA	Peptide anticoagulant	[43]
	Ornithodorin		Thrombin		[44]
Ornithodoros savignyi	Savignin	14 kDa	Thrombin	Peptide	[32]
	Tap-like protein	7 kDa	FXA	Peptide anticoagulant	[45]
	BSAP1–BSAP2		TF pathway inhibitor		[46]
Ixodes scapularis	Ixolaris	14 kDa	TF pathway inhibitor	Protein	[47]
	Salp14	15 kDa	TF pathway inhibitor	Protein	[48]
	TIX-5	7 kDa	Inhibitor FXa-mediated FV activation	Factor X inhibitor	[49]
Ixodes ricinus	Ir-CPI	3–4 kDa	Intrinsic pathway, fibrinolysis	Peptide	[50]
Amblyomma variegatum	Variegin		Thrombin	Lipocalin	[51]
Amblyomma americanum	FXa inhibitor		FXa inhibitor		[52]
	Americanin		Thrombin		[41]
Amblyomma cajennense	Amblyommine-X	7 kDa	FXa	RGD peptide	[53]
Haemaphysalis longicornis	Madanin 1 and 2	7 kDa	Thrombin	Peptide	[40]
	Haemaphysalin		FxII/XIIa		[54]
	Longistatin	23 kDa	Fibrinolysis	Peptide	[55]
Rhipicephalus appendiculatus	65 kDa protein	65 kDa	Factor Xa inhibitor	Prothrombinase complex	[56]
Rhipicephalus (Boophilus) microplus	BmAP	14 kDa	Thrombin	Anticoagulant protein	[42]
	Boophilin	1.7 kDa	Thrombin, trypsin, plasmin	Protein	[57]

	Microphilin	1.8 kDa	Thrombin	Protein	[58]
Boophilus calcaratus	Calcaratin	14.5 kDa	Thrombin	Protein	[59]
Dermacentor andersoni	Inhibitor of FV and FVII		Inhibitor of FV and FVII		[60]
Hyalomma truncatum	FXa inhibitor		FXa inhibitor		[61]
Complement inhibitors					
Ornithodoros moubata	OmCI	16.8 kDa	C5, prevention of interaction of C5 with C5 convertase	Complement inhibitor	[62]
I. scapularis	Salp20	48 kDa	Interacts with C3 convertase		[6]
	Isac	18.5 kDa	Alternative complement pathway, interacts with C3 convertase		[6]
I. ricinus	IRAC I, II, Isac paralogues		Alternative complement pathway, interacts with C3 convertase		[63]
Immunosuppression/immunomodulation					
Ixodes scapularis	Salp15	15 kDa	Impairs IL-2 production and T-cell proliferation, binds *B. burgdorferi* OspC, protects the spirochete from antibody-mediated killing	Protein	[64, 65]
	IL-2-binding protein		Inhibits proliferation of human T cells and CTLL-2 cells		[66]
	ISL 929 et ISL 1373	10 kDa	Impair adherence of polymorphonuclear leukocytes	Protein	[67]

(continued)

Table 1.1 (continued)

Tick species	Molecule	Molecular weight	Target and/or function	Nature of biochemical	References
	Sialostatin L, L 2	12.5 kDa	Inhibits cathepsin L activity	Protein	[68]
Ixodes ricinus	Iris	43 kDa	Iris modulates T lymphocyte and macrophage responsiveness, induces Th2-type responses	Protein	[69, 70]
	Bip		Inhibitor of B-cell proliferation	Protein	[71]
	Ir-LBP		Neutrophil	Protein	[72]
Dermacentor andersoni	P36	36 kDa	T-cell inhibitor	Protein	[73]
Hyalomma asiaticum	BIF	13 kDa	Inhibits LPS-induced proliferation of B cells	Protein	[74]
	Hyalomin A, B		B suppresses host inflammatory responses (modulation of cytokine secretion, detoxification of free radicals)	Peptide	[75]
Rhipicephalus appendiculatus	Japanin	17.7 kDa	Reprogrammes DC responses	Protein	[76]
Dermacentor reticulatus	SHBP	22 kDa	Histamine- and serotonin-binding protein	Protein	[77]
Rhipicephalus appendiculatus	RaHBP(M), RaHBP(F)	21 kDa	Histamine-binding proteins	Protein	[78]
Rhipicephalus appendiculatus	TdPI	13.5 kDa	Tryptase inhibitor	Peptidase	[79]
Amblyomma americanum	MIF	17 kDa	Inhibitor of macrophage migration	Protein	[80]
Rhipicephalus sanguineus	Ado, PGE2	352.4 kDa	Modulate host inflammatory responses	Saturated fatty acid	[81]

Chemokine binding					
Rhipicephalus sanguineus	Evasin-1	10.46 kDa	Chemokines CCL3, CCL4, CCL18, CXCL1	Protein	[82, 83]
	Evasin-3,	7 kDa			
	Evasin-4	12.03 kDa	CCL5 et CCL11		
Wound healing, angiogenesis					
Ixodes ricinus	Metalloprotease		Inhibits angiogenesis	Enzyme	[84]
Haemaphysalis longicornis	Haemangin		Inhibits angiogenesis	Protein	[85]
	HLTnI; troponin I-like molecule		Inhibits angiogenesis	Protein	[86]

1.2.1.1 Tick Vasodilators

Ticks are able to disarm the vasoconstriction branch of the hemostatic system by the presence of salivary vasodilators. The latest discovered are molecules which increase blood flow by antagonizing vasoconstrictors produced by the hemostatic system following tissue injury.

All known tick salivary vasodilators reported to date are nonproteinaceous vasodilatory compounds. They include lipid derivatives such as prostacyclin and prostaglandins [20, 87]. Examples of salivary vasodilators (Table 1.1) from the hard tick, *Ixodes scapularis*, are a salivary arachidonic acid lipid derivative prostacyclin [20] and prostaglandin E2 (PGE2) [36]. The latest molecule is a short-acting vasodilator and also an inhibitor of platelet aggregation that exerts its effect by increasing cyclic adenosine monophosphate (cAMP) in smooth muscle cells resulting is vasorelaxation. The saliva of the lone star tick, *Amblyomma americanum*, also contains the vasodilator PGE2 and, additionally, PGF2a [23, 24]. The presence of PGE2 has also been reported in *Rhipicephalus* (formerly *Boophilus*) *microplus* [25], *Haemaphysalis longicornis*, and *I. holocyclus* [88]. However, a tick histamine release factor (tHRF), secreted in *I. scapularis* saliva [21], and a novel *I. ricinus* serine proteinase inhibitor (serpin), IRS-2, which inhibits cathepsin G and chymase [22], probably also act as modulators of vascular permeability [8].

1.2.1.2 Tick Inhibitors of Platelet Aggregation

Platelet aggregation represents the initial and most immediate stage of defense to avoid blood loss during tissue injury (hemostasis). Following vascular injury, platelets adhere to the subendothelial tissue and then become activated by agonists such as collagen, thrombin, adenosine diphosphate (ADP), and thromboxane A2. Agonists bind to specific receptors on the surface of platelets and initiate a long and highly complex chain of intracellular chemical reactions that lead to platelet aggregation to form the platelet plug, promote clotting, and release vasoconstrictor substances. The platelet aggregation cascade is targeted by ticks at several stages [Table 1.1; 18]. The ability of ticks to counteract the platelet aggregation cascade occurs in several stages [89]. Thus, ticks target ADP via salivary apyrase, which hydrolyzes the phosphodiester bonds of ATP and ADP or inhibits ADP-induced platelet aggregation [31], or prevent activation of platelets by collagen [28, 38]. Interaction between fibrinogen and the GPIIb–IIIa complex is the important final step to platelet aggregation.

Integrin αIIbβ3 (glycoprotein IIb–IIIa, GPIIb–IIIa) is an inactive receptor on resting platelets which, when activated, regulates aggregation and adhesion of platelets [90]. This glycoprotein receptor binds fibrinogen resulting in a platelet–fibrinogen–platelet interaction or platelet aggregation by fibrinogen cross-linking. ADP secreted by activated platelets provokes integrin and Ca2+–dependent platelet aggregation. Thrombin, ADP, and adrenalin increase the receptor affinity to their ligands (plasma protein, fibrinogen, and von Willebrand factor), which are responsible for binding

to platelets during aggregation. Accordingly, tick saliva contains disintegrin-like peptides that block the binding of adhesive proteins to GPIIb–IIIa receptor [30, 39], therefore inhibiting the platelet–fibrinogen–platelet interaction, even if platelets are activated [29, 30, 39]. This antiplatelet strategy is used by the soft ticks, *Ornithodoros moubata* and *O. savignyi*. These ticks contain proteins named disagregin (7 kDa) and savignygrin, respectively, which bind to GPIIb–IIIa in platelets [29, 31]. Disagregin uses a motif that is different from known GPIIb–IIIa antagonists to bind to the receptor, whereas savignygrin uses the classical Arg–Gly–Asp (RGD) motif to bind to GPIIb–IIIa. The saliva of the hard tick, *Dermacentor variabilis*, contains a protein named Variabilin (4.9 kDa) which has an RGD motif and blocks this receptor. However, this peptide has little sequence homology to other GPIIb–IIIa antagonists [39]. Additionally, Ixodegrins from *I. pacificus* and *I. scapularis* display sequence similarity to Variabilin, with two additional cysteines in the RGD position [37], but their disintegrin activity has yet to be confirmed [89].

It is interesting to note that when different tick species use the same strategy to counteract a biological activity, they may still use different proteins. However, in addition to the previously described integrin *O. moubata* produces Moubatin (17 kDa), a salivary antiplatelet factor which belongs to the lipocalin family of beta-barrel structures that, in general, bind small hydrophobic molecules [34, 91]. Salivary proteins with lipocalin structure have also been described in the tick, *Rhipicephalus appendiculatus* [78]. In addition, activation of platelets by collagen is prevented, for example, by Moubatin, a specific inhibitor of collagen stimulated platelet activation from *O. moubata*, whereas tick adhesion inhibitor (TAI) identified in the same tick species inhibits the adhesion of platelets to matrix collagen [28, 35]. Another inhibitor of collagen-mediated platelet aggregation, Longicornin, was isolated from the hard tick *Haemaphysalis longicornis* [38]. However, Longicornin does not bind directly to collagen fibers and does not affect platelet adhesion to collagen, indicating that the inhibitor, similarly to Moubatin, shares a common receptor with collagen.

The strategy used by most blood feeders to block platelet aggregation is to destroy or hydrolyze the platelet agonist ADP. This is achieved by the presence of the salivary enzyme, apyrase (EC 3.6.1.5), which hydrolyses the phosphodiester bonds of nucleoside triphosphates and diphosphates but not monophosphates. Apyrase activity has been reported in the saliva of many ticks including *I. scapularis*, *O. moubata* [33, 36], and *O. savignyi* [92]. Apyrase from *R. microplus* belongs to the 5'-nucleotidase family [93]. On the other hand, apyrase activity has not been detected in the saliva of, for example, *A. americanum* [23], but increased prostaglandin levels in the saliva of this tick inhibit platelet aggregation by preventing ADP secretion during platelet activation [23, 94].

Thrombin, the protease activated at the end of the blood coagulation cascade, is a potent agonist of platelet activation. Salivary antithrombins from soft ticks, including *O. moubata* and *O. savignyi*, have been characterized as anticoagulants as well as inhibitors of platelet aggregation induced by thrombin [32, 95]. The serpin IRS-2 from *I. ricinus* inhibits both cathepsin G- and thrombin-induced platelet aggregations [22].

1.2.1.3 Tick Inhibitors of the Blood Coagulation Cascade

Blood coagulation involves a series of enzymatic reactions whereby an inactive proenzyme (coagulation factor) is converted to an active form, which then activates the next proenzyme in the series. Thrombin is involved in the final common pathway of the coagulation cascade, which in turn cleaves fibrinogen into fibrin. Polymerization of fibrin results in blood clot formation. A number of inhibitors of serine proteases involved in the coagulation cascade are the most characterized entities from the saliva of ticks. Anticoagulants from ticks can be classified based on four mechanisms of action: thrombin inhibitors, inhibitors of activated factor X (FXa), inhibitors of the extrinsic tenase complex (ETC), and contact system protein inhibitors [96], with thrombin and FXa being the most common targets.

Inhibitors of Thrombin

A strategy employed by ticks to inhibit the blood coagulation cascade is to block thrombin activity. Thrombin is the last enzyme in the blood coagulation cascade and is a strong agonist for platelet aggregation. Several specific direct thrombin inhibitors with various modes of action have been characterized in the salivary glands of both soft and hard ticks [32, 40, 42, 52, 59, 95, 164; Table 1.1]. Variegin, characterized from *A. variegatum*, has structural similarity to, but is much more potent than, hirulog, a 20-amino-acid synthetic thrombin inhibitor based on the natural leech peptide hirudin [51]. In addition, Boophiline [57] and Rhipilin-1 [97] have been described in the saliva of the ticks *R. microplus* and *R. haemaphysaloides*, respectively. Americanin, the salivary antithrombin from *A. americanum*, is a specific, reversible, and a slow tight-binding inhibitor of thrombin [52]. The salivary antithrombin from *O. savignyi* is a 12.4 kDa protein named Savignin [32] which is a slow, tight-binding inhibitor of thrombin and interacts with the active site as well as with the binding exosite of this protease [30]. Savignin is 63 % identical to Ornithodorin, the salivary antithrombin from *O. moubata* [44]. Soft tick antithrombins insert their N-terminal residues into the thrombin active site inhibiting the activity of this protease, whereas traditional Kunitz-type inhibitors use a central, reactive loop. In addition, various other peptides with protease-inhibiting activity, such as Microphilin [58] and anticoagulant protein (BmAP) [42] from *R. microplus* or Calcaratin [59] from *Boophilus calcaratus*, are not ranked in any of the previous groups.

Inhibitors of Factor Xa

The tick anticoagulant peptide (TAP) from the saliva of the soft tick, *O. moubata*, is the most intensively studied soft tick anticoagulant [43]. TAP has some homology with Kunitz-type inhibitors, with a molecular mass of 6.977 kDa, but is a highly

specific, reversible competitive inhibitor of factor X activation [FXa; 62]. FXa is involved in the activation of thrombin, hence, the importance of blocking the activity of this protease for hematophagous arthropods. TAP binds FXa with a dissociation constant of 180 pM. The soft tick, *O. savignyi*, also contains an FXa inhibitor with 46 % identity to TAP [45]. Moreover, FXa inhibitors are reported from the saliva of the lone star tick, *A. americanum* [41], and from the saliva of *Hyalomma truncatum* [61]. Amblyomin-X recombinant protein derived from an *A. cajennense* transcript encoding a protein containing an N-terminal Kunitz-type domain and a C-terminus with no homology to any known sequences was also found to inhibit FXa [53]. Salp14, a protein belonging to the salivary protein (Salp) family, was identified in saliva of *I. scapularis* and specifically inhibits the FXa active site [48, 98].

Two anticoagulants have been identified from the salivary glands of the tick, *O. savignyi*. These two anticoagulants, termed BSAP1 and BSAP2, have molecular masses of 9.3 and 9.2 kDa, respectively, and are inhibitors of the extrinsic pathway of the blood coagulation cascade [46]; no sequence information is available for them yet. An anticoagulant from *R. appendiculatus* saliva probably targets components of the prothrombinase complex different from FXa [56]. Inhibitors of FV and FVII have been described for *D. andersoni* [60].

Inhibitors of the Extrinsic Tenase Complex (ETC)

Ixolaris, a tissue factor (TF) pathway inhibitor belonging to a novel group of tick anticoagulants, was isolated from *I. scapularis* [47, 99]. Ixolaris is a small protein (9.8 kDa) of 140 amino acids containing ten cysteines and two Kunitz-type domains [47]. It inhibits the intrinsic pathway and shows homology to Salp14 and Salp9Pac, also present in saliva of *I. scapularis*. Recombinant Salp14 prolongs activated partial thromboplastin time (APTT) and specifically inhibits factor Xa [48]. These proteins probably belong to a novel family of anticoagulants with related functions.

Inhibitors of Protein Contact System

Rhipicephalus microplus trypsin inhibitor-A (BmTI-A) is a kallikrein and elastase inhibitor of the BPTI–Kunitz type [100]. The inhibitor increases APTT but does not prolong prothrombin time (PT) or thrombin time (TT). In addition, a plasma kallikrein–kinin system inhibitor named haemaphysalin was identified in *H. longicornis* [54]. This inhibitor interferes with reciprocal activation between factor XII and prekallikrein. A contact phase inhibitor (Ir-CPI) present in *I. ricinus* salivary glands inhibits the intrinsic coagulation pathway and, to a much lesser extent, fibrinolysis in vitro [50].

1.2.2 Additional Tick Salivary Anti-hemostatic Activities

Many biological activities which may be related to host hemostasis have been described in tick saliva.

A fibrinolytic activity has been detected in *I. scapularis* saliva which is due to the presence of a metalloprotease. The role of salivary metalloproteases in tick feeding appears to be related to their antifibrinogen- and antifibrin-specific activities [101]. These proteolytic activities are metal dependent and target gelatin, fibrin, fibrinogen, and fibronectin but not collagen or laminin. These activities may confer additional anticoagulant activity by preventing the formation of the fibrin clot or dissolving the already formed blood clot. Kunitz-type serine proteinase inhibitors (RsTI, 8–18 kDa) were isolated from the larvae of *R. sanguineus* [102]. Their role in hemostasis is predicted to be similar to serine proteinase inhibitors such as those found, for example, in *R. microplus* [100], and they target plasmin and neutrophil elastase.

Serine protease inhibitors with similarity to the insect serpin family have also been discovered in ticks [103, 104]. Tick serpins might also interact with host defense responses, including hemostasis.

Calcium-binding proteins belonging to the calreticulin family are also present in tick saliva. They may play a modulating role in host hemostasis through binding calcium ions required as coagulation enzyme cofactors [105].

Phospholipase A2, most probably responsible for the hemolytic activity of saliva, has been detected in *A. americanum* [106]. This salivary activity hydrolyzes arachidonyl phosphatidylcholine and is activated by submicromolar calcium. It has been suggested that this phospholipase (55 kDa) may be involved in producing PGE2 from host substrates and that it may also be responsible for the hemolytic activity reported in *A. americanum* saliva [107].

Ixodes scapularis saliva has been reported to inhibit key pro-inflammatory activities of neutrophils such as aggregation following activation by anaphylatoxins, the release of enzymes, production of oxygen radicals, or the phagocytosis of bacteria [108].

Moreover, anti-IL-8 activity was reported from the saliva of *D. reticulatus*, *A. variegatum*, *R. appendiculatus*, *H. inermis*, and *I. ricinus* [109].

Histamine is a highly potent inflammatory mediator and a vasoactive factor which binds to H1 and H2 receptors, causing edema and erythema by dilating and increasing the permeability of small blood vessels. Additionally, histamine is a regulator of the T-cell response [110]. *Rhipicephalus appendiculatus* has a set of novel salivary histamine-binding proteins with a lipocalin structure [78]. Interestingly, *R. appendiculatus* histamine-binding proteins are beta-barrel structures with two binding sites instead of one binding site for hydrophobic molecules. Similar proteins were identified in salivary glands of *I. scapularis* [111] and *A. americanum* [112].

Another mediator of the inflammatory response, serotonin, is secreted by tissue mast cells (in rodents) and has similar activities to histamine. A serotonin-binding protein (22 kDa) was isolated from *D. reticulatus* salivary glands [77]. This protein

is similar in structure to the *R. appendiculatus* histamine-binding protein with two binding sites, one of which binds histamine, while the other is slightly larger and is able to accommodate and bind serotonin [165].

Ixodes scapularis has a salivary protein that specifically inhibits the alternative pathway of the complement cascade [113]. Isac (*I. scapularis* anticomplement) is an 18 kDa protein that inhibits the formation of C3 convertase, which acts as a regulator of the complement cascade [6]. However, the sequence of Isac is not homologous to any known complement cascade regulator. Longistatin, a plasminogen activator identified recently in *H. longicornis*, was found to hydrolyze fibrinogen and delay fibrin clot formation [55].

1.2.3 Tick Compounds and Angiogenesis

Angiogenesis is characterized by the invasion, migration, and proliferation of smooth muscle and endothelial cells, a process that involves sprouting of new capillaries from existing blood vessels. It is a highly regulated process, essentially in many physiologic conditions, including development, reproduction, and wound repair. Vascular cell adhesion molecules appear to contribute to its regulation, and several pathologic conditions have been related to unregulated angiogenesis, as in tumor development [114]. Disintegrins have been characterized as platelet aggregation inhibitors that can prevent adhesion of tumor cell lines to extracellular matrix (ECM) components. Saliva from *I. scapularis* has been reported as a potent inhibitor of angiogenesis [115]. Relatively few disintegrins have been molecularly cloned and expressed (Table 1.2); therefore, salivary disintegrin inhibitors of angiogenesis remain a relatively unexplored field of investigation with great promise for a range of medical applications.

Ticks are the most important source of disintegrins among arthropods. In fact, ticks must inhibit the interaction of cells with ECM components during the long feeding period as part of the mechanism by which they keep blood flowing through its proboscis. Below we detail the salivary disintegrins which have been characterized molecularly or functionally in ticks. These disintegrins are described in Table 1.2.

1.2.3.1 Disintegrins and Functions

Variabilin

Variabilin is present in the SGs of the hard tick *Dermacentor variabilis* and inhibits platelet aggregation induced by ADP (IC_{50} ~ 150 nM), collagen, and thrombin receptor peptide SFLLRNP. It also blocks platelet adhesion to fibrinogen. It is a potent antagonist of the fibrinogen receptor integrin αIIbβ3 and the vitronectin receptor αvβ3 [39].

Table 1.2 Tick salivary disintegrins [116]

Name [reference]	Tick species	Mol wt	IC50	R/S/P[a]	Tripeptide	Cell target	Integrin
Variabilin [39]	*D. variabilis*	5	157 nM	N/N/Y	RGD	+2.5	αIIbβ3
ISL929/1373 [29]	*Ixodes* sp.	10	[d]	Y/N/Y	[d]	Neutrophils	αMβ2[d]
Monogrin [117]	*A. monolakensis*	10	150 nM	Y/N/Y	RGD	Platelets	αIIβ3
Tick antiplatelet inhibitor (TAI) [35][b]	*O. moubata*	15	8 nM	N/N/Y	[d]	Platelets EC	α2β1, α1β1
Disagregin [29, 118]	*O. moubata*	6	104 nM	N/N/Y	RED	Platelets	αIIbβ3
Ixodegrin [37][c]	*Ixodes* sp.	7	[d]	N/N/N	RGD	Platelets[d]	αIIββ3
Savignygrin [30]	*O. savignyi*	7	130 nM	N/N/Y	RGD	Platelets	αIIbβ3

[a]*R* obtained in recombinant form, *S* structure available, *P* inhibition of cell function tested with recombinant or purified proteins, *Mol wt* molecular weight (approximate), *EC* endothelial cell, *NIF* neutrophil inhibitory factor, *HPI* hookworm platelet inhibitor, *TAI* tick adhesion inhibitor
[b]TAI has not been molecularly identified
[c]Ixodegrin has not been expressed or purified
[d]IC50, or integrin specificity unknown, or not confirmed

Disagregin

Disagregin is a 6 kDa protein from the SGs of *O. moubata* that potently blocks ADP-induced platelet aggregation (IC_{50} ~ 150 nM) [29]. In addition, disagregin inhibits platelet aggregation by different agonists, blocks platelet adhesion to fibrinogen, binds to resting and ADP-activated platelets, and also binds integrin αIIbβ3 in activated platelets with K_D ~ 40 nM. Cross-linking experiments also demonstrated binding of disagregin to integrin αIIbβ3. In contrast, disagregin does not affect endothelial cell adhesion to vitronectin, which is mediated by integrin αvβ3 [29].

Savignygrin

Savignygrin is a platelet aggregation inhibitor purified from the soft tick *O. savignyi* and is similar to disagregin. It inhibits platelet aggregation induced by ADP (IC_{50} ~ 130 nM), collagen, thrombin receptor-activating peptide, and epinephrine. It also blocks binding of α-CD41 to platelets, binding of αIIbβ3 to fibrinogen, and adhesion of platelets to fibrinogen, suggesting it targets the fibrinogen receptor. Savignygrin forms a complex with both αIIbβ3 subunits, and this complex formation is unaffected by the activation state. This disintegrin belongs to the BPTI family of serine protease inhibitors and presents the integrin RGD-recognition motif on the substrate-binding loop of the Kunitz fold [31]. Additionally, savignygrin can

promote disaggregation—which is an inhibition of platelet aggregation at a post-aggregation level—through occupation of the αIIbβ3 receptor. Savignygrin-like molecules have also been cloned from the soft tick *O. coriaceus* [115].

Monogrin

Monogrin was purified from the SGs of the soft tick *Argas monolakensis*. Both recombinant and purified monogrins block ADP-induced platelet aggregation (IC_{50} ~ 150 nM) but not initiation of shape change. Monogrins were found to interact with integrin αIIbβ3 by surface plasmon resonance [19].

Ixodegrin

This family was named after identification of *I. pacificus* [37] and *I. scapularis* putative cysteine-rich proteins with an RGD or KGD domain indicative of proteins that interfere with fibrinogen binding to platelets, acting as platelet aggregation inhibitors. Ixodegrins display sequence similarity to variabilin. Recently, a protein described from the SGs of the tick *Amblyomma variegatum* showed similarities to *I. scapularis* ixodegrins [15]. Ixodegrin remains to be produced in a heterologous system to confirm its functional activity.

Tick Antiplatelet Inhibitor (TAI)

TAI (~15 kDa) has been purified from *O. moubata* SGs but has not been molecularly cloned. It inhibits platelet adhesion to soluble collagen under static conditions (IC_{50} ~ 8 nM) without affecting the onset or maximum aggregation triggered by collagen or other platelet agonists. TAI also affects endothelial cell adhesion to collagen and has partial inhibitory activity for fibronectin-mediated platelet adhesion. Further, it outcompetes anti-α2β1 monoclonal antibody Gi9 binding to platelets, suggesting it is an integrin α2β1 antagonist [35].

ISL929/1373

ISL929 and ISL1373 are two *I. scapularis* salivary proteins that have been described as neutrophil inhibitors. Expression of both molecules is induced upon tick feeding and mostly expressed in the salivary gland. Recombinant ISL929 and ISL1373 appear to reduce expression of β2 integrins and to decrease production of superoxide by neutrophils in vitro. Furthermore, mice immunized with both proteins had increased number of neutrophils at the site of attachment, suggesting that they interfere with inflammation in vivo [15].

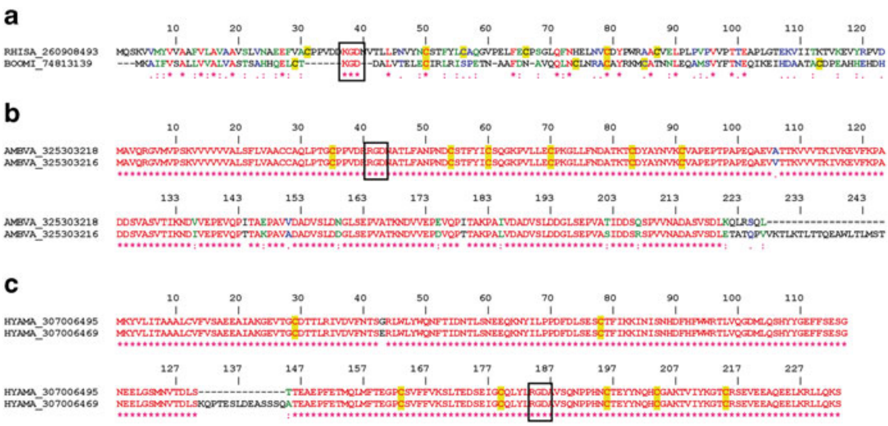


Fig. 1.1 ClustalW alignment for putative KGD (**a**) and RGD (**b** and **c**) disintegrins from metastriate ticks [116]

As described in Sect. 1.2.3.1, tick disintegrins exhibit substantial complexity and variability. In Sect. 1.2.3.2, we will focus on the structure of the different families of disintegrins, including both known and putative salivary disintegrins.

1.2.3.2 Disintegrins and Comparatives Structures

Disintegrins from Metastriate Ticks (*Dermacentor*, *Rhipicephalus*, and *Amblyomma*)

KGD and RGD Disintegrin Family

Two distinct KGD disintegrins from the salivary glands of *Rhipicephalus* ticks have been identified as mucin-like proteins (Fig. 1.1a). RGD disintegrins from *Amblyomma* sp. which have been identified as chitin-binding peritrophins (midgut protein) are shown in Fig. 1.1b. Figure 1.1c displays two sequences from *Haemaphysalis* sp. salivary gland which belong to the lipocalin family, one of them having an insertion between amino acids 130 and 145 [116]. Perhaps the RGD in these proteins is adapted for integrin recognition. *Dermacentor* sp. salivary glands are characterized by the presence of two highly related members, including 14 cysteines, and a KGD found between cysteines 10 and 11 [116]. Interestingly, a shorter sequence from *R. appendiculatus* was found to display a high degree of similarity to the other two members from *Dermacentor* sp.; the KGD is also located between two cysteines. Perhaps these molecules have evolved to interact with β3 integrins.

KTS/RTS Disintegrin Family

A family of KTS disintegrins has been found in *A. americanum* salivary gland (Fig. 1.2). These proteins belong to the Kunitz family of protein inhibitors. There are abundant transcripts coding for putative disintegrins with which the KTS tripeptide appears to

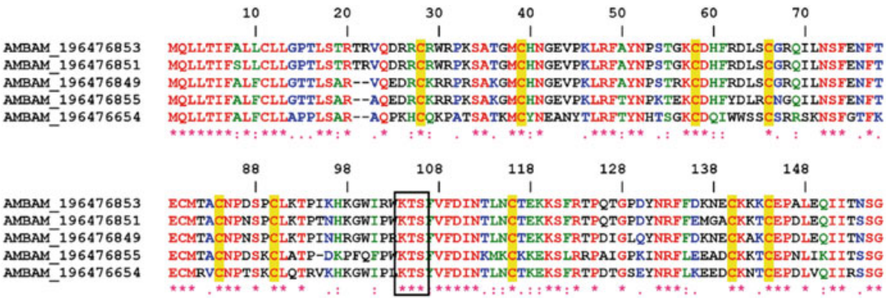


Fig. 1.2 ClustalW alignment for putative KTS disintegrins from *Amblyomma* sp. ticks [116]

be properly placed to interact with α1β1 integrins, assuming the specificity is the same as reported for the viperidae[1] KTS [119]. These molecules contribute to blocking endothelial cell adhesion to collagen and to assisting in the inhibition of angiogenesis and host response to injury [115]. Three other molecules with KTS or RTS motifs were, respectively, found in *Amblyomma* or *Rhipicephalus* sp. [116].

Duodegrins

Bioinformatic analysis identified several duodegrin sequences with more than one tripeptide motif. In some proteins, VGD and RTS motifs exist, while in others, RED and VGD motifs were identified [116]. These sequences code for cysteine-rich proteins of high molecular weight in the midgut of ticks and include the protein, BM86, which is used as a vaccine against tick infestation [120, 121]. While its function is unknown, it might be related to protection of the tick gut against host neutrophil attack.

Disintegrins from Prostriate Ticks (*Ixodes* sp.)

RGD, KGD, and VGD Disintegrin Family

Short proteins from *Ixodes* sp. which display a typical RGD flanked by cysteines 5 and 6 are shown in Fig. 1.3a. They have been classified as putative secreted salivary proteins, since they have no match to other proteins. Likewise, a second family of putative RGD secreted sialogenins is presented in Fig. 1.3b. In Fig. 1.3c, two related putative disintegrins are aligned, one of which (IXOSC_67083633) is named ixodegrin-2A [37]. In addition, KGD and VGD motifs, shown in Fig. 1.4a, were reported in ixodid ticks [116, 122].

[1] The Viperidae (vipers) are a family of venomous snakes found all over the world, except in Antarctica, Australia, New Zealand, Ireland, Madagascar, Hawaii, various other isolated islands, and north of the Arctic Circle.

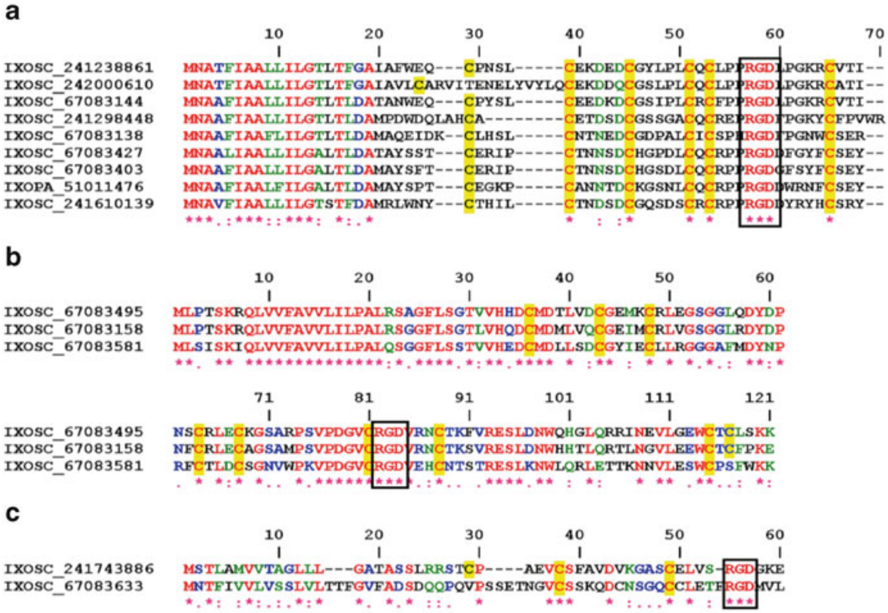


Fig. 1.3 ClustalW alignment for putative RGD (**a**) and RGD (**b** and **c**) disintegrins from Ixodidae ticks [116]

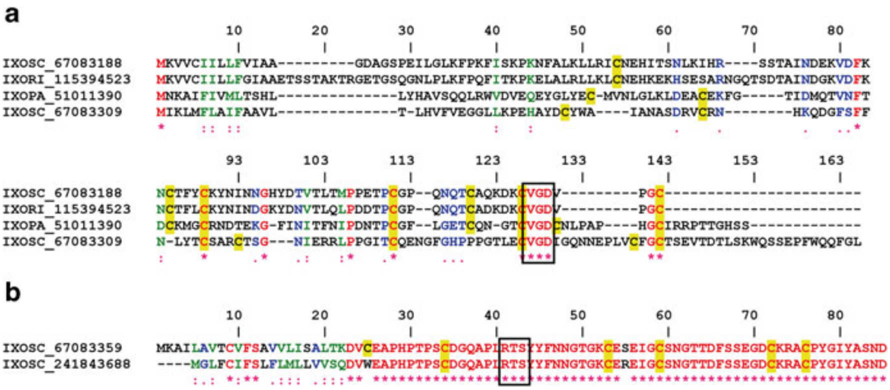


Fig. 1.4 ClustalW alignment for putative VGD (**a**) and RTS (**b**) disintegrins from Ixodidae ticks [116]

RTS Disintegrin Family

One sequence was found to display RTS motif properly flanked by cysteines, suggesting that this molecule might work as a disintegrin targeting α1β1 (Fig. 1.4b). This is a putative secreted protein without database hits. As reported for other RTS disintegrins, this sequence may also contribute to blockade of angiogenesis by tick

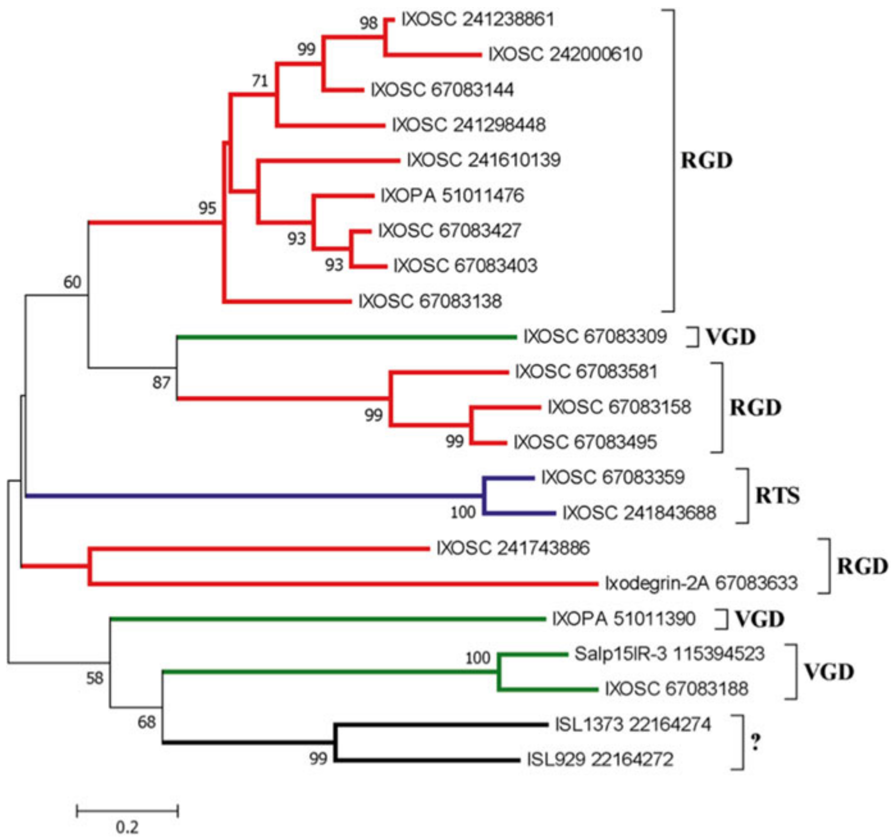


Fig. 1.5 Neighbor-joining phylogram for the Ixodidae sequences presented in Figs. 1.5 and 1.6. The numbers in the phylogram nodes indicate percent bootstrap support for the phylogeny. The bar at the bottom indicates 20 % amino acid divergence in the sequences [116]

saliva [115]. Figure 1.5 displays a phylogenetic tree containing several salivary disintegrins from Ixodidae. It is clear that they are positioned in a separate clade as different families.

Disintegrins from *Ornithodoros* sp.

The repertoire of anti-hemostatics in hard ticks which feed for several days differs significantly from those found in soft ticks [7, 15, 19, 37, 117, 123, 124]. Two short RGD disintegrins from *O. parkeri* (Fig. 1.6a) have been identified as savignygrin-like-1 and -2 [124]. They are likely platelet aggregation inhibitors. Figure 1.6b contains two other sequences containing RGD motifs from *O. coriaceus* [123] belong to the lipocalin family of proteins and have eight cysteines. It remains unclear whether they target platelets, neutrophils, or endothelial cells until they are obtained in recombinant form for further experimentation.

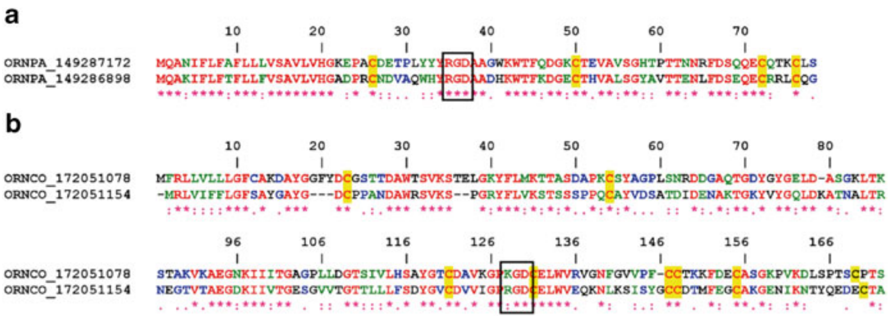


Fig. 1.6 ClustalW alignment for putative RGD (**a**) and K/RGD disintegrins, (**b**) from *Ornithodoros* ticks [116]

1.2.4 Tick Compounds and Host Immunity

The first lines of defense against invading pathogens are host cellular innate immune responses and the complement system. Tick salivary compounds can modulate both innate and acquired immunity of the hosts to protect themselves from inflammation and host immune responses [5, 16, 69, 125, 166]. Some hosts develop resistance to tick feeding, while others develop no protective immunity to tick infestations. Thus, host resistance or susceptibility depends on the tick–host association and can most likely be explained by tick-induced modulation of the host cytokine network [126, 127]. Repeated tick infestations and salivary gland extracts are known to suppress production of macrophage pro-inflammatory cytokines and the secretion of Th1 cytokines, whereas they upregulate Th2 cytokines, indicating a Th2 polarization of the host immune response [128, 129].

Despite relatively extensive knowledge of tick-induced host immunomodulation, only a few active molecules have been identified and characterized in tick salivary glands [5, 6, 64, 69, 71, 73, 80, 82].

1.2.4.1 Innate Immune Responses

Normally, the consequences of prolonged feeding of an ectoparasite would be local inflammation and rejection. However, ticks produce compounds that inhibit the pro-inflammatory functions of most cells infiltrating the attachment site, like neutrophils [108], NK cells [130], macrophages [131], T cells [73, 132], and dendritic cells [133]. Moreover, tick saliva contains a variety of inhibitory activities directed against many pro-inflammatory cytokines such as IL-2 and chemokines (CCL2/MCP-1, CCL3/MIP-1α, CCL5/RANTES, and CCL11/eotaxin) [127]. Evasins, a family of chemokine-binding proteins, have been detected in *R. sanguineus* ticks [71]. This family show selectivity to different chemokines: Evasin-1 binds to CCL3, CCL4, and CCL18; Evasin-3 binds to CXCL8 and CXCL1; and Evasin-4 binds to CCL5 and CCL11 [71, 81]. In addition, Evasin-3-like activities were described for

other metastriate tick species, which provide other evidence that ticks control host neutrophil functions during feeding.

A dipeptidyl carboxypeptidase activity was found to account for the salivary kininase activity of *I. scapularis* [134]. In fact, bradykinin and histamine are important mediators of itch and pain and can found to stimulate host grooming and removal of the feeding ticks. However, tick salivary kininases hydrolyze circulating kinins (e.g., bradykinin). Hard ticks also produce amine-binding proteins of the lipocalin family. A male-specific histamine-binding salivary protein (RaHBP(M)) and two female-specific histamine-binding salivary proteins (RaHBP(F)-1, 2) were isolated from the saliva of *R. appendiculatus* [78, 165], and the gene for a protein that binds both serotonin and histamine (SHBP) was identified in *D. reticulatus* [77]. In addition, a tick-derived protease inhibitor (TdPI) has been described and characterized from *R. appendiculatus* that suppresses the activity of human β-tryptases, mast cell-specific serine proteases with roles in inflammation and tissue remodeling [79]. Ticks also produce proteins that mimic host proteins to evade the host immune response [11]. A tick macrophage migration inhibitory factor (MIF) has been described in *A. americanum* [80]. It inhibits the migration of macrophages and most probably protects the tick from macrophage attack [11].

1.2.4.2 The Complement System

The complement system links the innate and adaptive responses of the host immune system and is activated via three main pathways (alternative, classical, and lectin pathway). The alternative pathway is the major line of defense against pathogens and ticks [11]. Several molecules with anticomplement activities were identified in tick salivary glands. Isac, Salp20, and Isac-1 from *I. scapularis* [6, 62] and the Isac paralogues IRAC I and II from *I. ricinus* [79, 135] inhibit specifically the formation of the C3 convertase of the alternative pathway by blocking binding of complement factor B to complement C3b. In addition, OmCI (*O. moubata* complement inhibitor) belonging to proteins of the lipocalin family has been the first natural complement inhibitor isolated from a soft tick that specifically targets the C5 activation step in the complement cascade [136; Table 1.1].

1.2.4.3 Acquired Immune Responses

A variety of tick species have been found to suppress in vitro proliferation of lymphocytes induced with T- and/or B-cell mitogens. Tick-induced immunosuppression of the host is also characterized by decreased primary antibody responses to T-cell-dependent antigens [11]. Moreover, ticks have evolved ways to alter production of T-lymphocyte cytokines. Generally, it has been reported that tick saliva polarizes the host immune response toward a Th2-type profile characterized by downregulation of Th1 cytokines (IL-2, IFN-γ) and enhanced production of Th2 cytokines (IL-4, IL-5, IL-6, IL-10, IL-13) [5, 137, 138]. The inhibition of T-cell

responsiveness to mitogens could result from the direct effect of salivary gland proteins on lymphocytes or from their production of IL-10, while upregulation of IL-4 and IL-10 probably leads to the development of a Th2 response [4, 132, 137, 138].

A 36 kDa protein (p36) present in the saliva of feeding *D. andersoni* has been characterized as a T-cell inhibitor [73; Table 1.1]. An immunosuppressor, Iris, was detected in *I. ricinus* females [69]. Iris suppresses T-lymphocyte proliferation, induces a Th2-type immune response, and inhibits the production of pro-inflammatory cytokines (IL-6 and TNF-alpha). A 15 kDa salivary gland protein from *I. scapularis* (Salp15) is another feeding-induced protein that inhibits the activation of T cells. Salp15 specifically binds to the CD4 molecules on CD4+ T (helper) cells, which results in inhibition of T-cell receptor-mediated signaling, leading to reduced IL-2 production and impaired T-cell proliferation [64, 122]. A secreted IL-2-binding protein that suppresses T-cell proliferation and the activity of other immune effector cells responsive to IL-2 stimulation was detected in the saliva of *I. scapularis* [66]. Sialostatin L, a protein with inhibitory action against cathepsin L that displays anti-inflammatory properties and inhibits proliferation of cytotoxic T lymphocytes, was also found in the saliva of *I. scapularis* [68].

B-cell inhibitory proteins (BIP and BIF) were identified in *I. ricinus* and *Hyalomma asiaticum asiaticum*, respectively [71, 74]. Apart from substances modulating the host immune responses, ticks also produce immunoglobulin-binding proteins that protect them primarily from ingested host immunoglobulins [139].

1.2.5 Tick Saliva and Pathogen Transmission

Pathogens (bacteria, viruses, piroplasms, etc.) are transmitted from the salivary glands of the tick to the host via salivary fluid. Some pathogens co-injected with saliva may be more infective because the blood feeders' saliva changes the physiology of the host at the feeding site by injecting an array of bioactive molecules. In addition, anti-inflammatory mechanisms may also enhance the transmission of tick-borne pathogens [136, 137]. It is suggested that various tick salivary compounds may have competing activities during infestation, and the amount of saliva injected may also influence tick feeding and pathogen transmission [129, 139; Table 1.3]. The saliva of the tick, *D. reticulatus*, promoted vesicular stomatitis virus growth in vitro [162], while the saliva of *D. reticulatus*, *I. ricinus*, and *R. appendiculatus* enhanced tick-borne encephalitis virus transmission [141]. In another example, the saliva of the tick, *R. appendiculatus*, enhanced *Thogotovirus* transmission [140] and, in combination with interleukin-2, increased *Theileria parva* infection in lymphocytes [163]. *I. ricinus* saliva increased bacteremia (*Borrelia afzelii*) in C3H mice [142] and exacerbated the proliferation of the bacterium *Francisella tularensis* in mice [146]. Finally, there is evidence that the pathogen *B. burgdorferi* in *I. scapularis* might use Salp15 during transmission to a vertebrate host, as it specifically interacts with *B. burgdorferi* outer surface protein C, and the binding of Salp15 protects *B. burgdorferi* from antibody-mediated killing in vitro [65].

Table 1.3 Examples of saliva-assisted transmission (SAT) of tick-borne pathogens [11]

Pathogen	Tick species	SAT factor	Effect	References
THOV	*Rhipicephalus appendiculatus*	SGE[a]	Enhanced transmission and infectivity	[140]
TBEV	*Ixodes ricinus*	SGE	Enhanced transmission and infectivity	[141]
Borrelia afzelii	*I. ricinus*	SGE	Accelerating effect on spirochete proliferation in the host, suppression of pro-inflammatory cytokines	[142]
Borrelia burgdorferi s.s.	*I. ricinus*	SGE	Accelerating effect on spirochete proliferation in the host	[143]
B. burgdorferi s.s.	*I. ricinus*	Saliva	Increased spirochete load in host skin, increased transmission to ticks	[144]
Borrelia lusitaniae	*I. ricinus*	SG[b] lysate	Increase of spirochete loads in target organs	[145]
B. burgdorferi s.s.	*I. scapularis*	SG lysate	Increase of spirochete loads in target organs	[145]
Francisella tularensis	*I. ricinus*	SGE	Accelerates proliferation of the bacteria in the host	[146]
THOV	*R. appendiculatus*		Non-viremic transmission	[147]
TBEV	*I. ricinus*		Non-viremic transmission	[148]
Borrelia afzelii	*I. ricinus*		Co-feeding transmission	[149]
B. burgdorferi s.s.	*I. ricinus*		Co-feeding transmission	[150]
B. burgdorferi s.s.	*I. scapularis*		Co-feeding transmission	[151]
TBEV	*I. ricinus*	Saliva	In vitro modulation of infection rate of DCs[c] and production of cytokines	[152]
B. afzelii	*I. ricinus*	SGE	Anti-inflammatory activities	[153]
B. afzelii	*I. ricinus*	SGE	Impairment of signal pathways in DCs	[154, 155]
		SGE	Impairment of DC functions	[156]
B. burgdorferi	*I. ricinus*	Tick feeding	Modulation of skin innate immunity	[157]
	I. ricinus		BIP inhibition of B lymphocyte proliferation induced by *B. burgdorferi* lipoproteins OspA and OspC	[158]
B. burgdorferi	*I. ricinus*		Salp15 Iric-1, a Salp15 homologue, binds to OspC of *B. burgdorferi* s.s., *B. garinii*, and *B. afzelii*	[159]

(continued)

Table 1.3 (continued)

Pathogen	Tick species	SAT factor	Effect	References
B. burgdorferi	*I. scapularis*		Salp15, immunosuppressive functions, binds to OspC of *B. burgdorferi*, protects the spirochete from antibody-mediated killing, facilitates transmission and replication of the spirochete	[65]
			Salp25D, antioxidant, facilitates the acquisition of spirochetes by the vector from an infected mammalian host	[160]
			Salp20, inhibits complement, facilitates pathogen survival	[6]
			P8, lectin complement pathway inhibitor, facilitates pathogen transmission	[161]
Anaplasma phagocytophilum	*I. scapularis*		Salp16, facilitates migration of the pathogen to salivary glands	[171]

[a]*SGE* salivary gland extract
[b]*SG* salivary gland
[c]*Dcs* dendritic cells

1.3 Conclusion

The area of tick saliva research has taken a great leap forward in recent years. Molecular biology and high-throughput approaches are increasing our knowledge of the proteins present in the salivary glands of ticks. This new information together with the vast knowledge acquired over the last three decades on the pharmacology of tick saliva and immune responses to tick salivary proteins has the potential to open new venues to the understanding of tick saliva on blood feeding and pathogen transmission. Understanding of the molecular basis of the strategies used by ticks to evade host resistance and immune mechanisms that lead to host protection offers great promise to engender new strategies for the use of tick salivary antigens as vaccines to control vector-borne diseases.

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Chapter 2
Qualitative and Quantitative Proteomics Methods for the Analysis of the *Anopheles gambiae* Mosquito Proteome

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Abstract *Anopheles gambiae* is the major African malaria vector. Insecticide and drug resistance highlight the need for novel malaria control strategies. *A. gambiae* exhibits daily (diel and/or circadian) rhythms in physiology and behavior that include flight, mating, sugar and blood-meal feeding, and oviposition. Olfaction is important for detecting blood-feeding hosts, sugar feeding sources, and oviposition sites. We previously reported on mRNA array-based changes in gene expression under light–dark cycle (diel) and constant dark (circadian) conditions. We were able to characterize 25 known or putative olfactory genes in female heads. We sought to follow up on these reported changes in gene expression and correlate them with expected changes in protein response. Here, we describe our recently developed methods and meta-level results for both qualitative and differential proteomics analyses of *A. gambiae* mosquitoes collected in a time-of-day-specific framework to assess temporal changes in protein abundance over the 24-h day. The traditional challenges associated with proteomics are amplified in an insect such as the mosquito, which contains a large amount of non-proteinaceous material associated with the cuticle and trachea and a high dynamic background of proteins associated with flight muscles and oxidative metabolism (e.g., myosin, glutathione S-transferases). We thus sought to use targeted, quantitative proteomics to directly measure differences in protein abundance in a time-of-day-dependent manner. We used multiple reaction monitoring (MRM), which has the advantage of being able to probe selected target lists with high sensitivity, wide dynamic range, and good/excellent reproducibility. We first characterized proteins in a qualitative format and subsequently examined subsets of specific proteins of interest in a high-fidelity quantifiable

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approach. Targeted quantitative multiple/single reaction monitoring (MRM/SRM) proteomics allowed for the measurement of changes in protein abundance in a time-of-day-specific manner over the 24-h diel cycle. Utilizing this accurate technique requires robust and reproducible protein/peptide preparation techniques in order to obtain consistent data. Here, we describe a technique using liquid N_2 homogenization-based protein extraction and proteolytic digestion applied to multiple discrete tissues (whole heads, antennae, total head appendages, compound eyes, and bodies), with subsequent liquid chromatography/tandem mass spectrometry (LC/MS/MS)-based analysis of the resulting tryptic peptides. This technique is largely portable and should function well in any arthropod system with little modification. The results of our analyses are the generation of tissue-discrete determination of peptides, targeted quantitative analysis of peptides, and the deposition of datasets in VectorBase.org for use by the vector biology, arthropod, and proteomics research communities.

Abbreviations

ABC	Ammonium bicarbonate, Ambic
BEH	Ethylene-bridged hybrid
CID/CAD	Collision-induced dissociation/collisionally activated dissociation
CV	Coefficient of variation
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
FASP	Filter-aided sample prep
FDR	False discovery rate
IAA	Iodoacetamide
LC/MS/MS	Liquid chromatography tandem mass spectrometry
MS-MS/MS	Mass spectrometry tandem mass spectrometry
MRM/SRM	Multiple reaction monitoring/selected reaction monitoring
NaDOC	Sodium deoxycholate
OBP	Odorant-binding protein
ORF	Open reading frame
PCR	Polymerase chain reaction
PMSF	Phenylmethylsulfonyl fluoride
Q1, Q2, etc.	First and second quadrupoles
QqQ	Triple quadrupole
RT	Retention time
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TCEP	Tris(2-carboxyethyl)phosphine hydrochloride
TFE	2,2,2-Trifluoroethanol
THAs	Total head appendages
TIC	Total ion current

TOP8,10	Top 8,10 precursor selection method
UHPLC	Ultrahigh-performance liquid chromatography
XIC/EIC	Extracted ion chromatogram
ZT	Zeitgeber time

2.1 Introduction

Substantial improvements in biological separations coupled to mass spectrometry and expanded access to annotated genome sequences have facilitated the use of proteomics on otherwise difficult or uncultured organisms. Sample preparation then remains the key component in the generation of high-quality reproducible proteomics results. Robust protocols have been developed for many types of samples such as formalin-fixed paraffin-embedded tissue, plant-based materials, and samples recovered from acrylamide gels [1–5]. Other improvements in sample preparation for mass spectrometry-based proteomics include "universal" sample preparation techniques with micelle exchange detergent extraction, aprotic solvent protein extraction, and acid-labile detergents [6, 7].

Arthropod proteomics presents unique challenges in sample extraction. The exoskeleton chitin is not readily extracted or solubilized. Previous efforts to extract proteins specifically from *Anopheles* mosquitoes have used aqueous buffers, which bypass somewhat the losses in quality from dominant components, or sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by in-gel digestion–extraction to separate them [8]. These approaches have not been exhaustively tested for reproducibility and by design miss many partially soluble proteins and membrane-bound or membrane-associated polypeptides. Using SDS-PAGE as a primary extraction method has poor quantitative performance compared to in-solution analysis [5]. Harsher extraction procedures might be applied but run the risk of contaminating the protein fraction with the non-proteinaceous and/or insoluble material. It is crucial that any study undertaking the comparison of multiple independent samples across a study generates similar uncontaminated and biologically quantifiable protein distributions.

One desirable feature in carrying out proteomics of large sample sets is to generate a sample that is capable of being analyzed using a single dimension of reverse-phase liquid chromatography. Quantification of biological change is more practical when the targets are not distributed among many fractions. Furthermore, the number of analyses is limited to the sample (x) and the number of technical replicates. Straightforward experimental courses like the one described here (Fig. 2.1) with just seven time points required ~1 week of instrumental time per tissue. Multidimensional proteomics separations can easily generate 10–30 fractions for analysis per time point, each requiring technical and biological replication. Thus, developing a procedure that is effective under single dimension analysis is ideal. Here, we describe a protocol that takes advantage of the "natural fractionation" provided by distinct

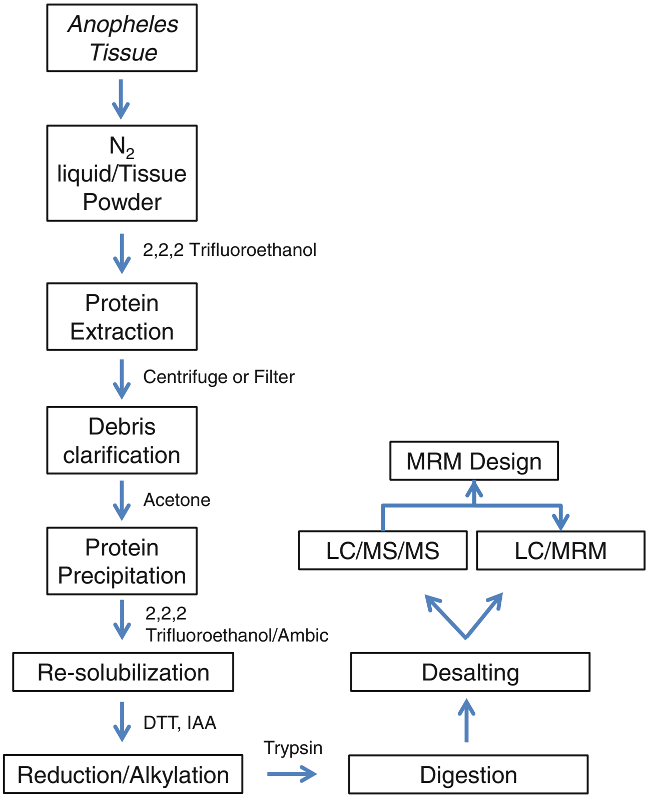


Fig. 2.1 Flowchart of protein extraction method for *A. gambiae* mosquitoes. There are notable differences compared to a traditional lysate-proteome extraction and analysis. These include the double aprotic extraction and resolubilization in 2,2,2 Trifluoroethanol (*TFE*); and for sample reduction an extended time with increased concentration of reducing agents dithiothreitol (*DTT*) or tris(2-carboxyethyl)phosphine hydrochloride (*TCEP*) prior to digestion with trypsin for subsequent LC/MS/MS analysis

tissue types in the *Anopheles* mosquito (e.g., antennae, heads, eyes, bodies (thorax and abdomen), and total head appendages (THAs; i.e., maxillary palps, antennae, and proboscis)) [9]. This requires only a numerical increase in the number of samples to analyze and provides tissue-specific detection and quantification of protein species. Ultimately, when surveying or targeting an arthropod proteome, we sought a robust, generic extraction that balances efficient, reproducible, and stoichiometric protein recovery without excessive contamination by exoskeleton components.

Anopheles gambiae Giles (Diptera: Culicidae) is the major African malaria vector. The developing insecticide and malarial drug resistance highlights the need for novel malaria control strategies [10, 11]. Our research interest is in the biology of the *A. gambiae* mosquito in a spatial and temporal context, i.e., through tissue-/organ-specific and time-of-day-specific analyses. *A. gambiae* exhibits daily rhythms in physiology and behavior that include flight activity, dusk mating, sugar and

blood-meal feeding, oviposition, host odorant sensitivity, and susceptibility to pesticide challenge [9, 12–16]. These rhythms are driven exclusively or in part by an endogenous circadian ("about a day") clock, which regulates daily rhythms in biochemistry, physiology, and behavior. The circadian clock is cell autonomous and at the molecular level is comprised of a series of transcriptional–translational feedback loops, completion of which takes approximately 24 h [17]. An improved understanding of mosquito chronobiology will yield insights into developing novel control strategies for this important disease vector.

We previously reported a mRNA microarray-based 48-h analysis of circadian (observed under constant dark [DD] conditions) and diel (observed under light–dark [LD] cycle conditions) gene expression [18, 19], identifying >2000 rhythmically expressed *A. gambiae* genes (www3.nd.edu/~bioclock). Many of these genes, which cover diverse biological processes such as transcription/translation, metabolism, detoxification, olfaction, vision, cuticle regulation, and immunity, are regulated by the coordinated action of the endogenous circadian clock and the environmental LD cycle [18]. This highlights the fundamental roles that both the circadian clock and light play in the physiology of the mosquito.

In an effort to understand how these rhythms in gene expression code for functional changes in physiology and behavior, we have begun to utilize proteomic approaches to both *identify* protein species and *quantify* their abundance in a *tissue-specific* manner. Olfaction is important for detection of blood-feeding hosts, sugar feeding sources, and oviposition sites [20, 21]. Our initial experiments focused on olfactory tissues (antennae and maxillary palps) and how changes in chemosensory protein abundance might contribute to temporal changes in sensitivity to chemical odorants and correlate with day–night changes in mosquito feeding behavior [9]. This chapter describes our approaches associated with this experimental study [9] and expands upon it through the analysis of additional *A. gambiae* mosquito tissues, namely, head, eye, and body (thorax and abdomen).

2.1.1 Proteomics in Arthropods

"Bottom-up" proteomics refers to the determination of proteins based on the fragmentation and identification of peptides derived from those proteins. This typically involves digestion of the protein populations with enzymes such as trypsin, which cleaves proteins into peptides at Lys and Arg residues with high efficiency. Peptides generated in this manner are typically subjected to one or more rounds of liquid chromatography coupled to tandem mass spectrometry. Fragmentation patterns of the separated peptides are then compared to virtual libraries of theoretical digests of the assembled genome sequence, and protein identification is inferred from statistical matching of these data [22]. Although there are numerous ways to extract some quantitative information from these kinds of experiments, they are largely considered qualitative in nature owing to the stochastic manner in which the data are

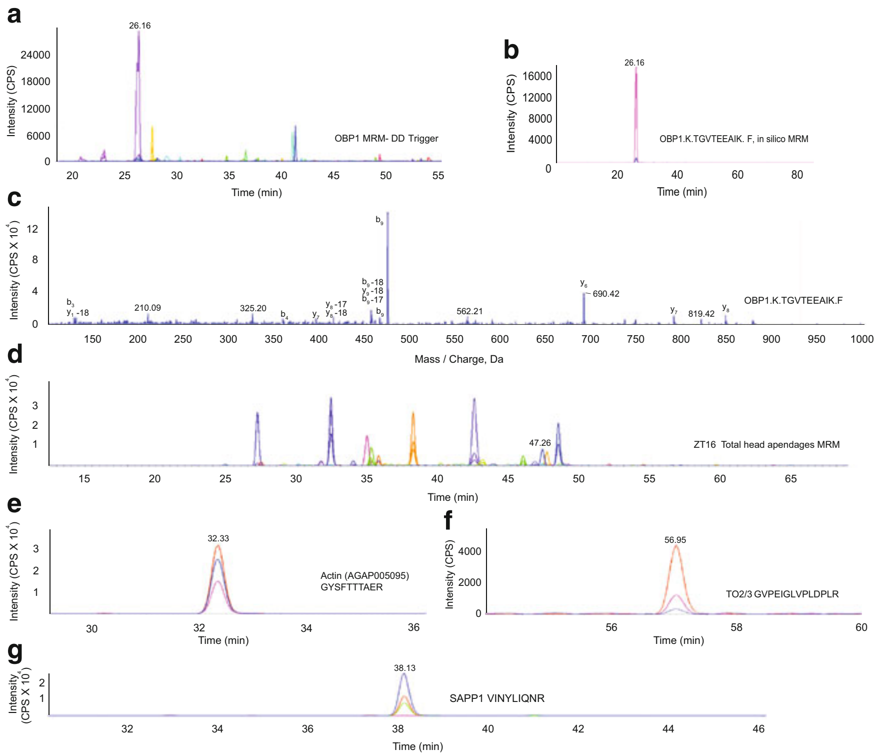


Fig. 2.2 MRM-determined transitions. Shown here is an example of an analysis from an *in silico* prediction of peptides derived from odorant-binding protein 1 (OBP1; AGAP003309) and subsequent MRM acquisition for all peptide ions. (**a**) Total ion current (TIC) for all MRM transitions detected in the experimental run. (**b**) A single transition extracted for sequencing. (**c**) Annotated spectrum of K.TGVTEEAIK.F (M+H)2+ 474.3 m/z. Highlighted are all matched b- and y-type ions. The transitions at 690.4 y6, 789.4 y7, and 589.3 y5 were used in subsequent MRM analysis. In (**d**–**g**) are example chromatograms for the analysis of an entire set of antennae at a single ZT time point. (**d**) 205 transitions (the LC chromatogram) overlaid of mosquito-extracted proteins at the ZT16 time point from extracted THAs. (**e**) A representative high-abundance MRM signal (actin) [GYSFTTTAER]. (**f**) A representative lower-abundance MRM signal (Takeout 2, TO2/ Takeout 3, TO3) [GVPEIGLVPLDPLR]. Shown here are three confirmatory transitions, the most intense (red) is used for quantification, the remaining transitions (2 in this case) are qualifiers. (**g**) A peptide from SAPP1 [VINYLIQNR] which has an identical mass (60 mmu) to the actin peptide in (**e**), illustrating the specificity of this approach (Reprinted from data used in Rund et al. [9])

typically acquired. These data are extremely useful, however, as a census of the protein populations within organisms and as a means to evaluate changes in proteomics experimentation and approaches [23–25]. One common implementation of bottom-up proteomics is in the identification of protein bands from polyacrylamide gels and/or liquid chromatography tandem mass spectrometry (LC/MS/MS). This approach was recently used to identify sex-specific differences in the population of odorant proteins in *A. gambiae* [31]. Additionally, it has been used in *Anopheles* and

Culex mosquitoes and in other insects, including *Drosophila*, *Apis*, and others [27–34].

Non-gel-based or "gel-free" proteomics has largely supplanted acrylamide for the analysis of complex proteomes. Peptide separations directly coupled with mass spectrometry can interrogate several thousand peptide events in a short period of time [35, 36]. Using proteogenomic approaches, which eschew traditional database searching, to better detect novel transcriptional and translational events, the Pandey group recently identified ~6000 protein features in mosquitoes [8]. These data represent individual, partially validated spectra for peptides corresponding to proteins detected in these organisms. Such empirical libraries of peptide spectra are easily transferred between instruments, whereas results from massive orthogonal separations followed by LC/MS are not. Moreover, the peptide spectra themselves can be used to generate target lists of peptides in order to perform *targeted quantitative proteomics* using multiple reaction monitoring, selected reaction monitoring (MRM/SRM) approaches [37]. This kind of mass spectrometry is extremely sensitive, reproducible, and linear. With appropriate standard curves commonly generated with stable heavy isotope internal standards, absolute quantification of target proteins/peptides can be obtained [38–40]. These experiments are typically performed using triple-quadrupole instrumentation which has performance characteristics ideally suited to this kind of analysis. Primarily they are the rapid ability to cycle through MRM/SRM target lists, high transmission efficiency of selected parent and daughter ions, and a high linear dynamic range of sensitivity.

Although heavily dependent on background contamination and complexity, validated targets effectively function as quantitative replacements for Western blots whereby antibody to antigen detection is replaced with MRM-to-peptide detection. This MS approach is obviously beneficial in studies of non-model organisms such as the mosquito, for which published and commercial antisera are not readily available, even though they might allow traditional immunoblotting analysis to be routine. Figure 2.2 highlights an example of a spectrum and chromatograms obtained from these targeted proteomics approaches on *A. gambiae* protein extracts digested with trypsin. As is evident, MRM-based approaches are largely chromatographic, relying on the specificity of the target pair, retention time (RT), and peak area of the peptide for quantification. Bottom-up data generate complex chromatograms with thousands of underlying spectra (a single example is shown from the chromatogram in Fig. 2.2a) which are matched to peptides/proteins using database search software.

We identified protein target lists for quantifying MRM-based time-of-day changes in olfactory protein abundance by acquiring individual bottom-up proteomics data on *A. gambiae* THAs. This was done for two reasons: (1) to develop and control the extraction methods used to obtain analytical quality protein extracts and (2) to determine which olfaction-related and control proteins were readily identified from mosquito preparations. As described below, a peptide spectrum taken from existing online proteomics data or acquired in-house can be converted into a targeted MRM-based peptide transition merely by harvesting the monitored parent mass(es). This is followed by producing a collection of the most abundant, typically higher mass/charge (*m/z*) fragments from that parent upon collision-induced disso-

ciation/collisionally activated dissociation (CID/CAD), the most common form of fragmentation currently used. Utilizing one's own proteomics data, as we did here to generate our targeted peptide transition list, has the advantage of confirming that a specific peptide and protein sequence is actually observed and detectable under the conditions in the laboratory and with the separations and chromatography utilized. Transitions determined in this way from existing in-house or empirical data validate well and in general are indistinguishable from those derived from synthetic libraries [37, 41–43].

Identification of proteins from these data allowed us to generate a protein library with tissue-specific localization consisting of 1327 protein features and 9751 peptides features at a 5 % and 1 % false discovery rate (FDR), respectively, for the combined tissue preparations separated using unfractionated peptide digests. Since we were primarily interested in olfaction and sensory input, peptide libraries and proteome-based MRM data were not generated from mosquito bodies and are therefore not represented in these protein/peptide numbers. These results focused our proteomics on olfaction gene products identified within antennae, THAs, and heads for further targeted analysis. Total heads and THAs were utilized as a general pool of *A. gambiae* mosquito proteins for bottom-up determination as they are relatively easy to harvest and process [9]. We hypothesized that quantitative detection of changes in these proteins would be based on the time-of-day rhythmic changes observed in transcripts of these genes. We have subsequently expanded this work to include compound eyes and bodies (thorax and abdomen). In order for a broad community to have access to all of these tissue-specific data, the processed data as described in Rund et al. [9] and herein in this chapter and raw data have been made available to VectorBase and the PeptideAtlas [37] which are, respectively, vector biologist and proteomics-centric repositories. Division and availability of the results across two separate and distinct repositories ensure fidelity of the raw data, and also visibility to the appropriate research community, depending on the manner in which they engage with the biological or bioanalytical findings.

2.1.2 Multiple Reaction Monitoring (Targeted Proteomics)

Targeted proteomics via MRM/SRM was named 2012 Method of the Year by *Nature Methods* [44]. It has been applied extensively to tissues, cell cultures, and, to some extent, microorganisms, but its use in less common model systems remains low [24, 33, 34, 42, 43, 45–48]. Fundamentally, it serves to provide a signal that is sensitive and specific for the target being analyzed, in this case, protein-derived peptides. The MRM signal is the best method to obtain quantitative (accurate and precise) analytical data from proteomics-based mass spectrometry. This is achieved by the use of triple-quadrupole (QqQ) type mass spectrometers, which have the advantage of two separate mass analyzers capable of high-resolution mass isolation with a high-efficiency collision cell in between. They are typically coupled to high-performance liquid chromatography systems at various flow rates and have detectors which are well suited to generate a linear signal in response to increasing peptide

concentrations. This is achieved by fixing the quadrupoles on a single mass at a time whereby a mixture of peptide ions enters the instrument but only a single peptide is preselected for fragmentation in the first step of mass analysis (Q1). Therefore, any other peptides from different proteins or different parts of the protein do not pass through. The selected peptide is fragmented using collisionally activated dissociation (CAD; Q2), and all of the fragment ions simultaneously pass into the second mass analyzer (Q3), where only a single daughter ion at a time is allowed to pass through to the detector. Therefore, any fragment ions that did not derive from the parent due to having similar mass are not transmitted. The signal generated from this event is recorded and the next transition on the MRM target list is monitored. This sequence/series repeats until the target list is exhausted (2–3 s), and then it repeats until the entire chromatography run is complete. This process can occur very fast (typically 1–5 mS), and several hundreds of these parent–daughter pairs can be interrogated within the peak width of typical microscale/nano-chromatography. The result of this process is that a specific MRM pair for any given peptide would be monitored 8–12 times across a typical peak. This enables the analyte-specific reconstruction of a peak profile which is then integrated to perform quantification (Fig. 2.2). The chromatographic signal from these analyses is the only data recorded, and there are no *spectra* in the classical sense of the word in proteomics. As a consequence, the data files and processing are modest, and files from many hundreds of samples take up just a few megabytes (Mb) of storage space. By contrast, data files from large-scale proteomics experiments readily consume 10 Mb or more per minute of acquisition time.

Recent efforts by the Aebersold group (at the Institute of Molecular Systems Biology, Institute for Systems Biology, at ETH Zürich) and others have taken advantage of the very specific nature of MRM/SRMs to design specific MRM transitions (targets) for every open reading frame (ORF) within an organism. Their efforts have been very successful for yeast, *Drosophila melanogaster*, *Mycobacterium tuberculosis*, and others [26, 37, 43, 47, 49]. With the addition of stable heavy isotope standards, the linear quantification derived from MRM/SRM can be used to determine *absolute* protein abundances within cells and organisms by taking advantage of stable isotope dilution mass spectrometry [38, 39]. The parent mass and abundant daughter ion masses (*m/z*) for each peptide are a fundamental property of the ion, not the mass spectrometer, and as such the masses that describe a specific peptide/protein are largely portable. The MRM atlas and PeptideAtlas projects currently run by the Moritz and Aebersold groups at the Institute for Systems Biology store and warehouse MRM/SRM data; this allows for researchers to utilize the data and not regenerate the peptide transitions [37]. It is with this portability and open access in mind that we sought to design large-scale MRM-based experiments initially for olfaction-related proteins/tissues in *A. gambiae* and to deposit the data in both the MRM/Peptide Atlas (http://www.peptideatlas.org/PASS/PASS00300) and VectorBase (https://www.vectorbase.org/proteomes) as described above [9]. Furthermore, these databases have been expanded to include our more recent analyses and additional *A. gambiae* tissues (e.g., eyes).

2.2 Sample Preparation

A. gambiae Pimperena S form mosquitoes were maintained at 85 % relative humidity and 27 ± 1 °C on a 12-h/12-h LD cycle (11-h full light, 11-h darkness (0 lx), and 1-h dawn and 1-h dusk transitions). Time of day is reported in 24 h in Zeitgeber time (ZT), with ZT12 defined as time of lights off under the LD cycle, ZT0 defined as the end of the dawn transition, and ZT11 defined as the start of the dusk transition. Access to 20 % high-fructose corn syrup was provided *ad libitum*. At least 24 h prior to time course collections, mosquitoes were transferred to light-tight boxes inside of a temperature/humidity-controlled bay, thereby ensuring precise exposure to the LD cycle. Light levels inside the boxes reached a maximum of ~327 lx during the light phase of the LD cycle. Additional details on mosquitoes and conditions can be found in Rund et al. [9, 12].

A. gambiae adult female mosquitoes were harvested by dry ice freezing at precise times of the LD cycle, and individual frozen tissues were later isolated by dissection on dry ice. Samples utilized for bottom-up proteomics analysis were collected for head (ZT12, ZT16, ZT0–4), antennae (ZT12, ZT16), THAs (ZT12, ZT16), eyes (ZT4, ZT8), and bodies (thorax and abdomen, with wings and legs removed; ZT4–ZT20). Mosquitoes collected during the dark phase of the photoperiod (i.e., ZT12, ZT16, and ZT20) were harvested in the dark, thereby preventing any photic modification of proteins (e.g., stimulation of the compound eye). In several cases, time-specific collections were chosen to reflect times of the day when we anticipated the proteins of interest to be at their highest expression levels, thereby maximizing potential peptide discovery (e.g., odorant-binding proteins [OBPs] in antennae and THAs). This was based on the peak expression levels for the specific rhythmic genes of interest [18, 19] and inclusion of a possible temporal delay in the occurrence of peak protein abundance [9]. Tissue samples were pooled prior to protein extraction, specifically 20 heads, 40 antennae, 20 THAs, 10 eyes, or 7–9 bodies.

For samples subjected to targeted proteomics analysis, mosquitoes were collected every 4 h for 24 h while maintained under strict LD cycle conditions as described above and at Zeitgeber times consistent with our complementary microarray gene expression analysis [18, 19]. Three separate collections of 30 presumed mated but not blood-fed adult female 4–7-day-old mosquitoes were harvested. ZT0 and ZT24 samples correspond to the same time relative to the LD cycle (i.e., dawn).

Preparations of biological materials for the bottom-up (proteome census) and the MRM/SRM quantification were essentially identical; the samples were diverted to specific instruments as indicated. This general harvesting procedure can serve as a standard protocol for extractions bound for future targeted or bottom-up proteomics studies including additional fractionation/preparation as needed [9].

2.2.1 A. gambiae *Heads, Antennae, and Total Head Appendages (THAs)*

Mosquito antennae, heads, and THAs were individually collected and processed identically to above. After transfer to cryogenic-safe 1.5 ml microcentrifuge tubes (USA Scientific, Ocala, FL), they were immersed in liquid N_2 (Linde, Murray Hill, NJ) until frozen. Tissues were ground using pestles (USA Scientific) until reduced to a fine powder with vigorous pulverization and reexposure to N_2 as needed. Equal volumes of 2,2,2-trifluoro-ethanol (TFE) and 50 mM ammonium bicarbonate (ABC or Ambic) with 1 mM PMSF and 1 mM EDTA (Fluka, St. Louis, MO) were added along the sides of the pestle to wash adhered particles into the tube [50]. Approximately 200 µl of total solvent was used per tissue preparation (containing organs from ~10 to 20 adult mosquitoes). Samples were then vigorously vortexed, pulse centrifuged, and then extracted for 15 min on a shaker stage. Samples were clarified by centrifugation at 5,000 xg for 10 min and the liquid extract was decanted to a fresh tube. Note that antennae are extremely low density and do not readily pellet under these conditions. The residual insoluble antennal material was removed by non-pyogenic (0.45 µm) filters or carried through experimental steps as a contaminant but was eventually removed during desalting steps prior to LC/MS/MS analysis. There was very little difference in quantity or yield of peptides due to residual antennae; however, their presence would be detrimental to the chromatography used for LC/MS/MS. Samples next had their TFE volumes reduced to <20 % in a SpeedVac (Genevac, Stone Ridge, NY), and the extracted proteins were precipitated by chilling on ice, adding 1:100 of a 2 % sodium deoxycholate (NaDOC) solution in H_2O (Sigma, St. Louis, MO) followed by >6x volumes of ice-cold acetone. Samples were incubated at −20 °C for 1–3 h and then centrifuged for 10 min at 15,000 xg. The acetone was decanted and the pellet dried in a SpeedVac for ~10 min.

Dried pellets were resuspended in equal volumes (25 µl each) of TFE and 50 mM ABC in the presence of 25–50 mM dithiothreitol (DTT) (Sigma), incubated at 56 °C for 1 h, and then iodoacetamide (IAA) (Sigma) was added to a final concentration of 35–70 mM and then incubated at room temperature in the dark for 20 min. This step is required to fully denature and resolubilize the protein samples and to reduce and alkylate the cysteine residues prior to digestion. Following reduction/alkylation, the sample volumes were normalized to 500 µl in 50 mM ABC to dilute the TFE, and two additions of 500 ng–1 µg sequencing grade trypsin (Promega, Madison, WI) were added 2 h apart with incubation at 37 °C and subsequent incubation at 37 °C overnight. Samples were centrifuged after digestion to remove any insoluble material, quenched by the addition of 5 µl of trifluoroacetic acid (Sigma), and then dried in a SpeedVac. Controlling the volume in this last step is critical. It ensures that all samples are prepared at a constant volume/concentration, every LC/MS LC/MRM analysis represents identical amounts of protein/peptide material, and losses/adsorption are consistent [42, 51].

2.2.2 A. gambiae *Bodies*

A. gambiae mosquito bodies from ~10 adults were prepared identically to the THAs as described above; however, initial preparations had excessive actin and chitin/cuticular proteins which are abundant in legs and wings. Therefore, the bodies were de-winged and de-legged prior to cryo-pulverization. 50 mM DTT was included in the body preparations from the beginning to prevent excessive melanization; the higher concentration of reducing reagent was due to a higher than-typical abundance of thiol-containing gene products involved in detoxification (e.g., glutathione S-transferase-like). NaDOC/acetone extraction was performed twice with a resolubilization step containing 20 μl TFE and 100 mM ABC with vigorous vortexing. Unlike heads, antennae and THAs, body debris could not be carried through trypsin digestion without irreparably limiting the quality of the proteome. This is presumably due to loss of trypsin activity or excessive "nibbling" of actin/myosin adhering to the body parts, which saturates and suppresses the subsequent proteome. Therefore, the insoluble and non-extracted material was removed by two rounds of centrifugation at 15,000 xg prior to acetone extraction.

2.2.3 A. gambiae *Eyes*

To analyze *A. gambiae* eye proteins, we adapted published protocols in which *Drosophila* retina proteins were reliably isolated from acetone-dried heads [52, 53]. Ten mosquito heads were manually removed from bodies and immediately immersed in 1 ml ice-chilled ethanol. They were washed 3 × 5 min in 1 ml chilled acetone and left in acetone for 72 h at −20 °C. Compound eyes were dissected from the head using tungsten probes and forceps, and retina/eye tissue was extracted. In this protocol, it is also possible to remove the corneal layer, thereby generating a pure retina preparation. Mosquito eyes were cryo-pulverized in triplicate as described above for head tissue and then processed for proteomics using the full filter-aided sample prep (FASP) protocol for trypsin from the Mann lab [7].

2.3 Mass Spectrometry

2.3.1 Data-Dependent "Bottom-Up" Proteomics

Qualitative mass spectrometry was performed separately on pools of heads, antennae, THAs, eyes, and bodies for generation of reference and tissue-specific proteomes, for which heads, antennae, and THAs are described in [9]. Eye and body data were not used for subsequent MRM/SRM targeting but served to validate the sample preparation and the specific utility of it for their future analysis. The

protein–trypsin digests of each tissue were resuspended in 40 μl of 1 % formic acid (FA) (Thermo Fisher, San Jose, CA) and then desalted using C18 ZipTips (Millipore) according to manufacturer's directions, with three extractions from each sample. 15 % of this material (2 μl) was injected onto a 100 μm × 100 mm C18 BEH column (Waters, Billerica, MA) and separated over a 90-min gradient on a Waters nanoAcquity UHPLC (Waters) system running at 1 μl/min. A linear gradient of 2–35 % A–B was used (A = H_2O 0.1 % FA; B = Acetonitrile, 0.1 % FA). MS-MS/MS was performed on an LTQ–Velos Orbitrap running a TOP8,10 data-dependent method as described in [9, 23, 54]. All data files were acquired in triplicate.

2.3.2 Database Searching/Protein Identification

Acquisition files (.RAW) were converted into .mgf (mascot generic format) peak lists using the RAW2MSM script from the Mann lab [55]. Peak lists were subjected to database search using the Paragon algorithm within Protein Pilot 4.0 against a current release of the *A. gambiae* translated FASTA file (VectorBase release VB-2013-04). False discovery rate calculations were made using the target–decoy method according to Elias et al. [56] where a reversed (scrambled) decoy version of the identical FASTA is created and searched in parallel with the correct *A. gambiae* database [57, 58]. In this manner, any given peptide-spectral match is equally likely by chance to occur in the known "incorrect" database, and this random match rate is used to estimate when such events would be recorded in the correct genome database. The approximate incidence and rate of false peptide/protein matches can then be calculated. For the subsequent MRM data, false discovery rates for all selected proteins were substantially less than 0.1 %.

2.3.3 MRM Generation

The generation of MRM-based assays to detect and quantify peptides has been the source of considerable review [40, 41, 59–62]. In general, an MRM measures the ion flux of the product of a specific parent ion (peptide corresponding to a specific protein) versus time for multiple ions of that peptide nearly simultaneously. This process is typically performed in a few mS of time, which means that tens to hundreds of these individual ion-specific transitions can be monitored in a space less than the width of a chromatographic peak. This means that in an unbiased manner, each ion to be monitored can be conducted almost continuously such that each ion can be quantitatively described with 10–12 sampling points across its chromatographic elution.

Figure 2.1 highlights the process of generating these data, and Fig. 2.2 describes how individual data (MRM based) appear. In Fig. 2.2c, a typical peptide MS/MS spectrum reveals that an individual peptide [K.TGVTEEAIK.F] produces multiple

fragment ions corresponding to various lengths of amino acid fragments produced from the N (b type) and C (y type) termini. Each one of these represents a potential pair (parent m/z) and individual daughter ion that describes an MRM transition. The coincidence of two or more of these ions being generated and detected simultaneously is often unique for a peptide, as shown in Fig. 2.2b. Note the high signal–noise ratio and "cleanliness" of the peak even though the injected material contains tens of thousands of individual peptides. This process is iterated for each peptide observed by traditional proteomics or inferred from *in silico* sequence gazing. These MRM transitions are assembled as a single method which contains the parent–daughter pairs for each peptide for every protein to be analyzed. An example of a complete chromatogram for an *A. gambiae* mosquito sample is shown in Fig. 2.2d, with individual peptide responses highlighted in Fig. 2.2e, f, and g.

MRM data were curated using Microsoft Excel and Skyline (MacCoss Lab) combined with manual refinement as described below [63, 64]. Four to nine transitions were selected per peptide for up to four peptides per protein. Subsequent validation of these data was performed as described below by serial analysis of antennae and THAs samples and similar to that used for bottom-up proteomics. The final list of selected and confirmed peptides (confirmed by the presence of an integrated peak area with an S/N [signal-to-noise] ratio of ≥5:1) contained 214 MRM transitions. Due to their routine use in normalizing immunoblots, tubulins (e.g., AGAP010929) were used for correction of loading and analytical variance as described in Section 3.5. Uniquely here, tubulins were used for the correction of variances in time-of-day processing as well (see below).

2.3.4 Quantitative Targeted Proteomics Data Acquisition

These transition lists, which are the mass/charge pairs used to define each MRM, were curated and confirmed as described, and the assembled list was used to analyze each of the individual biologically independent time courses of *A. gambiae* antennae or THAs in triplicate. One of the underlying general aspects of MRM-based data is the inherent linearity of the results. A peptide is typically linear with respect to itself; therefore, the change in peak area of a peptide transition in otherwise normalized data is proportional to changes in the level of the underlying protein [38, 40, 65, 66]. There are caveats to these assumptions, but for large datasets, they are in general true [40, 42, 43, 49]. Individual quantitative responses can be validated by the use of stable heavy isotope dilution mass spectrometry where a known amount of a chemically identical but heavy isotope version of each peptide is mixed with the sample. This enables perfect comparison and determination of peak area for each peptide and provides a stringent level of quantification, including the determination of absolute amounts of protein/peptide present in the biological material [38, 47, 65]. Expert validation is usually sufficient for relative quantitation and area ratio measurements as described below [46].

For our study, MRM data were acquired on a QTrap 5500 (ABSciex, Redwood City, CA) running in triple-quadrupole mode similar to that published earlier [46, 54, 65, 67]. Resuspended samples were injected onto a 100 μm × 100 mm C18 BEH column (Waters) running at 550 nl/min on an Eksigent (Dublin, CA) 2D UHPLC nano-system. A 90-min gradient from 2 to 35 % acetonitrile was used (0.1 % FA). Quality control injections were performed every 12 injections (4 samples × 3 technical replicates) to confirm blanks (i.e., reduce carryover) and to ensure retention time (RT) reproducibility. Peak retention should be excellent throughout a quantitative acquisition in order for the data to be readily interpreted. For the 24-h diel time courses presented here, drift was <1–2 % over >100 h of total acquisition time. RT stability was corrected with the β-tubulin IS peptide [.FPGQLNADLR.] (2y8) which was 0.31 % CV for RT for all injections of antennae material. This is typical for well-controlled chromatography in nano-LC/MRM experiments.

2.3.5 Validation/MRM Data Processing

Here, validation was performed by using a derivative of the expert/guided methods described in [59, 62, 63]. The identification of the underlying peptides was facilitated by the positive presence of their identification in the bottom-up proteome and the use of multiple distinct tissue types allowing for comparison and correlation of RT, protein level, and extractions (cross correlation). Essentially, identification of a given protein by the presence of a specific peptide across multiple tissues, e.g., antennae, heads, and THAs, confirmed the RT and daughter ions for a given MRM. Circadian and diel behavior as compared to the mean levels for known proteins like β-tubulin (nonrhythmic) allowed us to determine which set of peptide ions was specific for a given protein in cases where ambiguity existed (<10 %) [9, 54]. These represented the "expert" pool of peptide features that required manual intervention to process, identify, and quantify as described below [62]. Additionally, each peptide had to meet several quality criteria including reproducible RTs and peak area ratios (peak area/internal standard area) and % coefficient of variation (σ/mean), as described here. MRM data as described above for antennae and THAs were integrated using MultiQuant (v2.1) (ABSciex) with a 3-point Gaussian smooth as described in [9, 46, 65]. MRM-based peaks were checked for RT stability, ensuring <2 % variability across all triplicate injections for each peptide (214 individual peptide transitions were finalized, measured, integrated, and quantified) in our published work [9]. Peptide areas were normalized to the average signal response for all peptides for a given experimental run, and then peptide area ratios (internal standard) were determined by dividing the peak area of the peptide of interest (quantifier ion, which is typically the most intense product ion) by the quantifier ion of the β-tubulin and/or actin peptide nearest in RT. This served to correct for total extraction and loading differences between preparations and minimize false quantitative differences due to changes in ion signal or absolute loading as compared to "real" change due to biological time-of-day variations.

2.3.6 Time-of-Day Normalization

The use of a time-of-day collection strategy that included one full day starting at dawn the first day (ZT0) and ending at dawn the second day (ZT24) results in two samples, ZT0 and ZT24, from the same time of day. This enabled us to correct for changes in protein level due to growth and generalized expression due to aging of the *A. gambiae* mosquitoes, whereby ZT0 and ZT24 samples exist essentially as a matched pair of time-shifted duplicates. Ideally, such samples should contain similar dynamic range changes in protein expression due to time-of-day effects; however, nominal differences in protein levels, loading, and growth do occur. These are corrected through the use of a median-fold normalization method [9]. In order to report and observe *only* changes due to time-of-day-specific events, normalization was performed against an internal standard protein, β-tubulin, to account for these differences. Actin was also used as a visual reference to specifically compare between ZT0 and ZT24 and to complement the use of tubulin normalization across the 24-h cycle. These corrected data are ultimately how fold change is assessed. Tubulins, due to their 24-h constitutive RNA and protein expression, are frequently used in insect circadian experiments as an immunoblot loading control, including butterfly antennae [68, 69]. For our experiments, β-tubulin (AGAP010929) was used as an internal standard for normalizing protein levels across the time-of-day studies, and actin was used within replicate injections of an individual time point. The β-tubulin peptide EIVHIQAGQ[C]GNQIGAK was used for antennae and YLTVAAVFR for THAs normalization [9].

2.3.7 Caveats of MRM

Like many other biological assays, MRM-based quantitative approaches have challenges [40, 59, 60, 62, 66]. With respect to arthropods, some of these are magnified. One common problem in targeted peptide quantification is the presence of *ex vivo* modified peptides for analysis [70–72]. Oxidation of Met, His, and Trp residues is common but somewhat stochastic in most bottom-up proteomics experiments. This affects the quality of protein identification very little as it can be accounted for as a change in mass (+16 Da) in database searching [22]. Since MRM-based detection is mass and peptide specific, a change of 16 Da in nominal mass would remove that peptide from the MRM ion window for detection of that specific protein. We observed in the analysis of peptides from *A. gambiae* mosquitoes that this process seems more specific to a particular sample or certain peptides and specifically those with His residues. This required several proteins for which His- or Met-containing peptides to be discounted because a particular ZT time-of-day sample was more oxidized than others, even though they were prepared in parallel. This is likely due to the complex and time-consuming manual processes that were needed for sample preparation and handling differences that occur within the multiple time points of a

biological time course. We are presently investigating chemical- and technique-based approaches to reduce and eliminate stochastic oxidation in arthropod proteomics preparations.

Like all direct measurement techniques, these experimental approaches have limitations. Using proteomics with single-dimensional tissue separation, we could not defensibly identify and/or quantify several olfactory receptors from antennae or THAs, including the obligatory co-receptor required for all odorant receptor function, Odorant Receptor Coreceptor Agam\Orco (AGAP002560). Since there is no amplification step in proteomics like polymerase chain reaction (PCR) amplification for DNA applications, low abundance and high dynamic range samples remain a continuous challenge. These problems can be overcome with increases in the front-end preparation of samples followed by extended acquisition times or fractionation [6, 24, 25, 73, 74]. These currently require exponential increases in cost and/or time, so striking a balance between desired density of identification/quantification and effort remains one of the driving factors in arthropod proteomics. Traditional immunoblotting approaches remain useful in these circumstances, as demonstrated in our efforts to characterize the protein expression of Agam\Orco in olfactory tissues [9].

2.4 Data/Proteomics Summary

2.4.1 Bottom-Up Protein Summary

Although arthropods present difficult analytical challenges in sample preparation and proteomics processing, as discussed above, the specific external tissues and body segments create a unique opportunity to expand the density of the proteome by harvesting and processing individual tissues separately and combining the data. This is in contrast to preparation of the entire organism followed by extensive orthogonal fractionation and large amounts of instrumental analysis. Individual tissues thus represent a term we call “natural fractionation” and can be analyzed with just serial increases in sample processing, not geometric increases as in multidimensional separations. This approach has the additional advantage of providing tissue-specific proteome identification and ultimately tissue-specific proteome quantification for more meaningful biological insight.

In order for natural fractionation to be effective, each tissue type must deliver a large percentage of unique protein identifications versus the expectation for the entire organism, or it is only useful as a tissue-specific isolation technique. In Fig. 2.3, we demonstrate that for just three specific tissues located within *A. gambiae* heads (total heads, antennae, and eyes), we observe substantial increases in protein identification by individually analyzing each tissue. The histograms in Fig. 2.3a and b highlight that most of the identifications gained from additional tissues are unique; the number of redundant identifications, a routine problem in proteomics

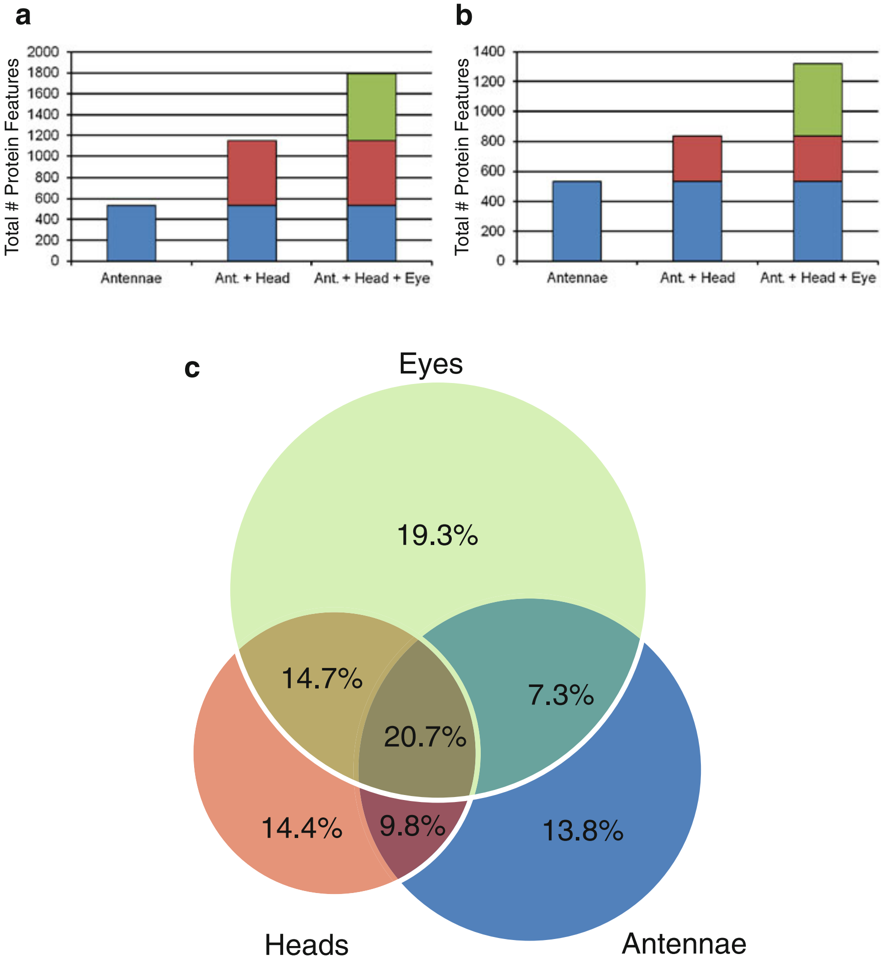


Fig. 2.3 Summary of *A. gambiae mosquito* tissue-specific contributions to the total proteome. (**a**) The cumulative distribution of the redundant *protein* identification at the 5 % FDR level. (**b**) Cumulative distribution of the nonredundant *protein* identifications at the 5 % FDR. Of note is the large ~100 % increase in unique identifications per specific tissue type. (**c**) Venn diagram of the individual distributions of the unique and shared identifications from each tissue type. Total non-redundant pool was 1322 identifications at a 5 % FDR. Only 20.7 % (273 proteins) from the total identifications were shared by all three tissues. THAs, which are part of the head, only added 5 additional protein identifications and are not included in this data presentation as they are nearly perfectly redundant with heads

experiments, increases at nearly identical proportion to unique (nonredundant) identifications. In total, this specific sub-dataset contains 1,322 nonredundant protein identifications at a 5 % FDR and 8,230 peptide identifications at a 1 % FDR. This represents one of the larger *A. gambiae* proteomes reported to date using existing public databases and further contains tissue-specific identification for several of the

dissectible organs from the head [8, 9]. Figure 2.3c illustrates how substantial the gains in unique identifications are per tissue. Just 20.7 % (273) of the 1,322 total protein identifications were identified in all three tissue types; other proteomes from tissues of similar location can identify 40–80 % of features in common; abundant cellular metabolism and chaperone genes dominate the shared pool in this and other tissue-specific proteomes [31, 45, 75–77]. These results can be compared to the other analyzed tissue fraction from heads, the THAs (not shown in Fig. 2.3), which show a nearly insignificant increase of 5 proteins, thereby generating a total proteome of 1,327 identified proteins. This indicates that the gains in identification are actually due to "fractionation/enrichment" and not merely sample amount, sample loading, or a larger pool of injections from which to curate protein identifications. The inclusive head-based proteome (i.e., total head, eye, antennae, and THAs) described for the THAs fraction included an additional 19 % peptide identification, but these mapped to only five (1,327 versus 1,322) additional protein features. The limited increase in protein number is partially due to the fact that the public database is not completely mapped, and the natural fractionation more completely describes the complete proteome.

Annotation of most vector genomes is incomplete and ongoing. It is crucial that the most current databases are utilized for proteomics search, as the search quality underlying proteomics is heavily dependent on high-quality assembled and translated FASTA files. The use of proteogenomic approaches, such as by the Pandey group on *Anopheles* mosquito, identified potentially thousands more peptide features than can be identified at the gene/protein level [8]. However, this analysis is not routinely implemented in most laboratories and utilizes multidimensional fractionations of in-gel digested, dissected *A. gambiae* mosquito(es) [8, 78]. As genome and translated FASTA files are improved for this organism, our existing data can be reprocessed to take advantage of gains in sequence completeness [79].

2.4.2 MRM Quantitative Protein Summary

We characterized a 24-h rhythmic pattern of gene expression from *A. gambiae* mosquitoes using MRM-based targeted protein quantification for the relative quantification of olfaction-related gene products [9, 19]. The biological relevance of our analysis of olfactory tissues is related to how mosquitoes smell and how this may change as a consequence of time-of-day changes in protein availability. A mosquito can detect odorants using its antennae and maxillary palps [80], and thus, we focused our time-of-day-specific analysis on these tissues, having isolated the antennae independently, and a tissue preparation of THAs that contained *both* olfactory tissues. We targeted 25 proteins of interest including control proteins, many of which were chemosensory proteins of the odorant-binding protein (OBP) class [81], as well as takeout proteins (TO1 [AGAP004263], TO2/TO3 [AGAP012703/AGAP004262]), that are associated with feeding behavior [82]. Our genomic analysis had indicated that many, but not all, of these targeted genes had rhythms that were phase concordant and peaking in expression around dusk (~ZT12).

Protein targets were selected based on previous gene-array rhythmic observations and based on detection using bottom-up proteomics as described above [9, 19]. The MRM-based targeted protein signals for olfaction-specific peptides were acquired over the 24-h time course and normalized both for amount of protein and to correct for nonrhythmic protein changes as described above. A selection of the results from this analysis as compared to mRNA-based expression data is shown in Fig. 2.4. The relative quantitative proteomics from the antennae fraction of *A. gambiae* mosquitoes as well as the corresponding mRNA expression profiles from whole-head preparations are compared (Fig. 2.4). Protein abundances were normal-

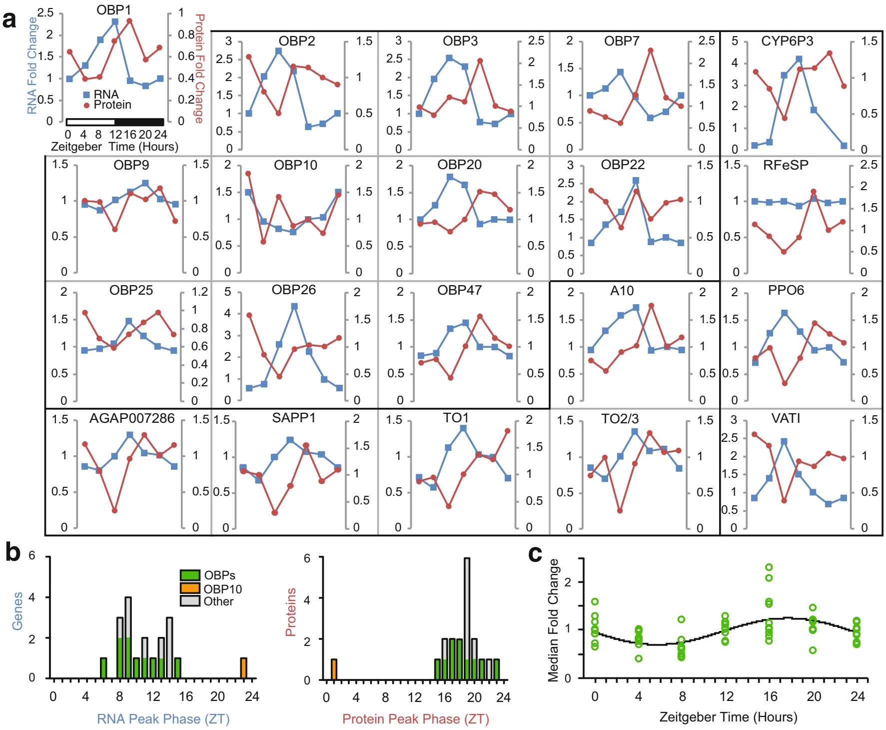


Fig. 2.4 (**a**) *A. gambiae* quantitative proteomics revealing rhythms in antennal protein abundance that correspond highly with RNA expression profiles from whole-head proteins are grouped into odorant-binding proteins (OBPs), non-OBP chemosensory proteins (AGAP007286, SAPP1, A10, TO1, and TO2/3), and non-olfactory proteins (CYP6P3, RFeSP, PPO6, and VATI). All gene symbols as listed in VectorBase (release VB-2013-04) except for the following genes: RFeSP (homologue to *Drosophila* Rieske iron–sulfur protein; AGAP008955), A10 (homologue to *Drosophila* antennal protein 10, AGAP008055), and AGAP007286 (*A. aegypti* OBP43 homologue). Note that with the most recent release of Vectorbase (VB-2014-08), the following *A. gambiae* genes have been updated and named: homologue to *Drosophila* RFeSP is now named ubiquinol-cytochrome c reductase iron-sulfur subunit; and homologue to *Drosophila* A10 is named chemosensory protein 2. Protein abundance normalized with tubulin. (**b**) Histogram of peak RNA expression phases in total heads and the corresponding peak antennal protein levels as determined by cosinor analysis from genes/proteins in *panel A*. (**c**) Cosinor analysis of antennae OBP protein levels from panel A ($p < 0.001$; acrophase ZT17.7); note OBP10 is excluded as it has antiphasic expression compared to the other OBPs (Reprinted from data used in Rund et al. [9])

ized similar to how Western blots are controlled using both total protein loading and normalization to β-tubulin [9, 44, 54, 60]. In addition to the robust nature of the protein quantification (% coefficient of variation, CV, typically <20 %), and the occurrence of time-of-day-specific changes in protein abundance for a majority of targets such as the OBPs and takeout proteins (the most obvious feature is that the majority of the olfaction-specific gene products detected had rhythmic profiles that shared a peak phase), occurring during the night (~ZT16). Furthermore, there was a substantial lag in peak signal between mRNA and protein, typically ~7 h for most of the proteins. There were a few cases in which the patterns of protein and corresponding RNA expression were notably different: for example, RFeSP (homologue to *Drosophila* RFeSP, now named *A. gambiae* ubiquinol-cytochrome c reductase iron-sulfur subunit; AGAP008955) had no apparent rhythm in RNA expression but exhibited a protein rhythm; whereas glutathione S-transferase E3 (GSTE3; AGAP009197) displayed rhythmic mRNA levels but corresponding constitutive protein levels [9]. Such delays between mRNA and protein rhythms have been reported in mammalian tissues, in addition to the nonoverlapping occurrence of circadian rhythms in gene expression versus protein rhythms [83, 84]. This highlights the importance of posttranscriptional and posttranslational modifications, including protein turnover, in shaping daily rhythms at the protein level.

Due to the high specificity, accuracy, and precision afforded by MRM-based detection, integration with additional molecular and behavioral analyses to correlate with protein levels can readily be performed and complemented with results using proteomics. This includes feeding behavior, electrophysiology, mating, and applications to vector–pathogen biology. Specifically, targeted detection of proteins by MRM in *A. gambiae* has been a powerful tool to generate immunoblot-like protein assays, which are multiplexed at the level of 20–50 total proteins per assay per tissue [9]. Expansion of the multiplexing is readily achieved with scheduled/stacked MRM methods, RT filtering, or duplicate injections with different multiplexed lists of MRM targets [62, 85]. Designing a multiplexed experiment requires substantially more materials, either for separate injections or to define RT precisely for stacked/scheduled MRM methods. In practice, this is not difficult, as a typical MRM-based assay described here consumes approximately 1 μg of total protein digest per injection, which is a 1–2 order of magnitude reduction compared to many Western blot procedures [9, 41, 44, 65]. This creates a protein array of quantitative differences that can be integrated with existing molecular (RNA based) and physiological/behavioral-based data acquired on isogenic organisms.

In terms of biological relevance, the specific targeted proteomics analysis described in this chapter (Fig. 2.4) was coupled with functional testing of the olfactory system, specifically electroantennogram analysis and testing of blood-feeding behavior preference [9]. The pre-dusk/dusk rhythmic peaks in chemosensory genes, in particular the *OBP*s and *takeout* gene expression, correspond with peak protein abundance at night and in turn coincide with the time of increased olfactory sensitivity to odorants requiring OBPs and times of increased blood-feeding behavior. This suggests an important role for OBPs in modulating temporal changes in odorant sensitivity, enabling the olfactory system to coordinate with the circadian/nocturnal niche of *A. gambiae*.

2.5 Conclusions and Future Perspectives

Protein identification in *A. gambiae* and other arthropods is a relatively new area in proteomics. Previous limits in instrumentation and robust preparation protocols have limited their use on these challenging samples. However, improvements and availability of genome sequences have expanded the use of these techniques for application of proteomics data to insect behavior and vector biology [8, 9, 78, 79, 86, 87]. Here, data generated from tissue-/organ-specific targeting and protein identification were applied to questions of a time-of-day-specific nature. We demonstrate an extensible set of procedures that enable the rapid, single step preparation of protein digest samples from *A. gambiae* mosquitoes that are capable of being simultaneously analyzed by both traditional bottom-up and targeted proteomics methods [9]. In particular, this requires careful attention to sample preparation in order to minimize chemical and handling modifications and contaminants that reduce the quantitative sensitivity of the data. Large quantitative and thorough sets of proteins can be generated by these methods using relatively modest amounts of instrument time with single-dimensional fractionation of the protein digests.

These data are readily available as an analytical resource for both vector researchers and proteomics-based studies. MRM-based data are highly portable, and the peptide-specific transitions and multiplexing will work with virtually no modification in any mass spectrometry laboratory. Further, the data are available through both VectorBase (https://www.vectorbase.org/proteomes) and the PeptideAtlas/MRM atlas (http://www.peptideatlas.org/PASS/PASS00300), as described earlier in this chapter [9, 37, 79]. We hope this dual model of contributing biologically focused analytical data will become routine among specific communities such as the vector biologists, chronobiologists, and bioanalytical chemists addressed here.

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Chapter 3
Lepidopteran Peritrophic Matrix Composition, Function, and Formation

Dwayne D. Hegedus, Umut Toprak, and Martin Erlandson

Abstract Lepidopteran larvae possess a robust digestive system featuring a multitude of hydrolytic enzymes that are able to accommodate an often highly polyphagous diet. Additional digestive complexity arises from the peritrophic matrix (PM) which encases the food bolus and compartmentalizes digestive processes. This review focuses on genomic and proteomic studies from several species that have identified what is likely to be the entire complement of proteins associated with the lepidopteran PM. In the process, a basal set of structural proteins common to the lepidopteran PM is described, and the roles of these proteins in PM structure and function are discussed. Finally, updated models for PM molecular architecture and formation which incorporate information about recently discovered proteins are provided.

Abbreviations

ALP	alkaline phosphatase
AMY	amylase
AST	astacin
CBD	chitin-binding domain
CDA	chitin deacetylase

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CHI	endo-chitinase
CHS-2	chitin synthase 2
CLECT	C-type lectin
CBP	chitin-binding protein
Ek	*Ephestia kuehniella*
GlcNAc	*N*-acetylglucosamine
GPI	glycosylphosphatidylinositol
β1,3GLU	β-1,3-glucanase
Ha	*Helicoverpa armigera*
IIL	insect intestinal lipase
IIM	insect intestinal mucin
Lsti	*Loxostege sticticalis*
mRNA	messenger RNA
Mc	*Mamestra configurata*
MD	mucin domain
NAG	*N*-acetylglucosaminidase
On	*Ostrinia nubilalis*
PAD	peritrophin-A domain
PBD	peritrophin-B domain
PCD	peritrophin-C domain
PM	peritrophic matrix
REPAT	response to pathogen
RNA	ribonucleic acid
Se	*Spodoptera exigua*
Tn	*Trichoplusia ni*

3.1 Introduction

In 1762, Lyonet [1] reported that the food bolus of lepidopteran larvae is encased within an acellular sheath. This structure has since been referred to as the peritrophic membrane [2], the peritrophic envelope [3], and the peritrophic matrix (PM). More than a century ago, Vignon [4] reviewed the current state of PM research, and Wigglesworth in 1930 [5] published the first compilation of histological reports on PM formation. The PM separates the midgut into two compartments: the endoperitrophic space which contains the food bolus surrounded by the PM and the ectoperitrophic space located between the PM and the midgut epithelium, though as outlined below the PM itself may be regarded as a distinct compartment. Waterhouse [6] suggested that the PM is intimately involved in insect digestion and several proven and interesting theoretical models as to how this may occur are being considered [7]. The PM is composed mainly of chitin, protein, and protein-associated carbohydrate in the form of glycoproteins, and the relationship between PM structure and

function has been reviewed several times [8–12]. The first PM-associated protein, a small chitin-binding glycoprotein, was isolated from the Australian sheep blowfly, *Lucilia cuprina* [13]. Wang and Granados [14] provided the first general molecular model of the lepidopteran PM which has been continually refined as additional structural components were identified [11, 12, 15].

This short review focuses on genomic and proteomic studies that have contributed to our understanding of the function, formation, and composition of the lepidopteran larval PM. We discuss the relationship between structure and function and provide new models for lepidopteran PM architecture and synthesis.

3.2 Peritrophic Matrix Composition

The PM is composed of chitin and proteins. Chitin is a polymer of *N*-acetylglucosamine (GlcNAc) and assembles into microfibrils. These congregate into larger chitin bundles and organize into a loose network to form the basal scaffold of the PM [16]. PM chitin is synthesized by the midgut-specific chitin synthase 2 (CHS-2), modified by enzymes such as chitin deacetylase (CDA), and degraded by chitinolytic enzymes, mainly endo-chitinase (CHI) and the exo-chitinase *N*-acetylglucosaminidase (NAG) [17, 18]. Structural PM proteins, called peritrophins, interact with the chitin scaffold and contribute structural and functional features to the PM such as integrity, elasticity, and permeability [12, 19]. One of the greatest advances toward understanding the structure and function of the PM has come from recent midgut genomic [20–28] and midgut and PM proteomic studies [15, 17, 25, 29–36] that have uncovered the full complement of PM-associated proteins from coleopterans, dipterans, and lepidopterans.

3.2.1 Classes of PM Proteins

PM proteins can be classified according to their functional characteristics into two broad groups, "structural PM proteins," the peritrophins, containing a chitin-binding domain (CBD) and "nonstructural PM proteins," which include hydrolytic (digestive) and chitin-modifying enzymes. Peritrophins have at least one CBD and can be extracted from the PM only with the use of strong denaturing agents [19]. Peritrophins are therefore integral PM proteins and interact directly with the chitin matrix. Most of the CBDs found in peritrophins have motifs with registers of six, eight, and ten cysteine residues which form three to five intra-domain disulfide bonds and are referred to as peritrophin-A, peritrophin-B, or peritrophin-C domains, respectively [19]. CBDs with an atypical eight-cysteine register referred to as a peritrophin-D domain are also found in some dipterans [12]. The peritrophin-A

domain (PAD) is ubiquitous among insects, whereas proteins with peritrophin-B (PBD) and peritrophin-C (PCD) domains are only present in dipteran larvae. The typical lepidopteran PAD is 48–57 residues long with six conserved cysteine residues. The consensus arrangement of the cysteines in PADs from lepidopteran peritrophins is $C^1X_{13\text{-}18}C^2X_5C^3X_{9\text{-}11}C^4X_{10\text{-}13}C^5X_{7\text{-}8}C^6$. Peritrophins may also possess one or more highly glycosylated mucin domains (MDs) which are referred to as insect intestinal mucins (IIMs) [37, 38].

Analysis of CBDs from putative PM proteins from a wide range of insect taxa did not reveal any strong phylogenetic relationships based on sequence alone [39]. However, examination of the underlying structure and organization of peritrophin CBDs and MDs allowed Toprak et al. [12] to propose a universal peritrophin classification scheme. Based on the latter analysis, four types of peritrophins are apparent: the simple, binary, complex, and repetitive types (Fig. 3.1).

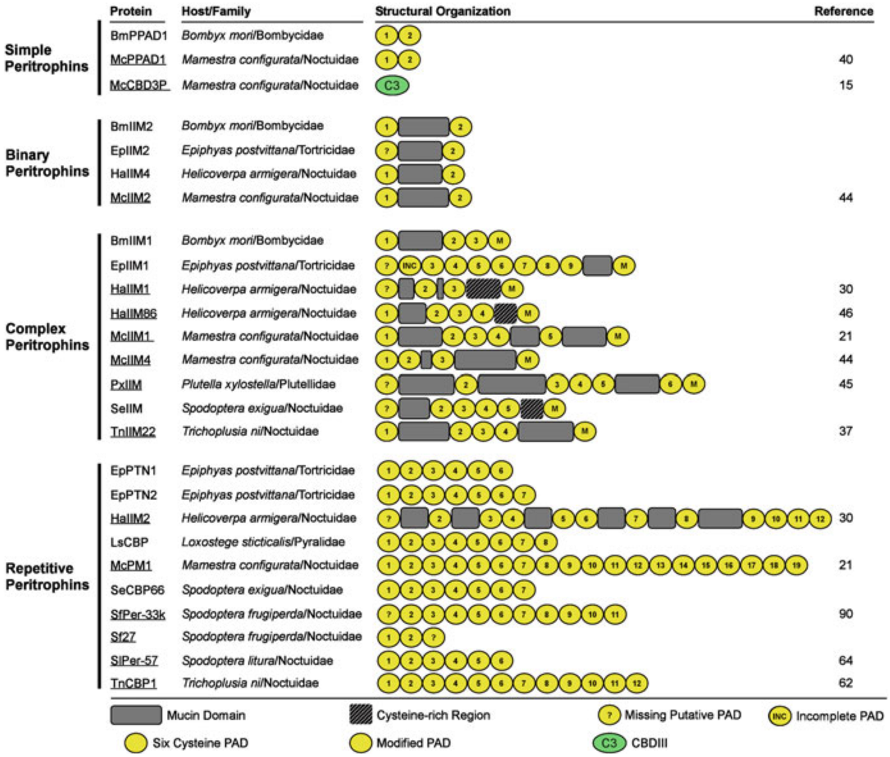


Fig. 3.1 Structural organization of representative lepidopteran peritrophins. The names of proteins demonstrated to be associated with the PM are underlined. *PAD* peritrophin-A domain, *CBD* chitin-binding domain

3.2.1.1 Simple Peritrophins

The simple peritrophins contain one or two CBDs and occasionally a small MD. They are present in lepidopterans, though they are more predominant and exhibit greater diversity with respect to the type of CBD in dipterans. Genes encoding simple peritrophins, such as McPPAD1, McPPAD2, and McPPAD3 from *Mamestra configurata* (bertha armyworm), are expressed in many tissues, including the midgut where they are expressed throughout larval development [40]. The PAD is a typical Type II carbohydrate-binding domain, but simple peritrophins with a single Type III carbohydrate-binding domain containing a register of ten cysteine residues ($C^1X_{12\text{-}13}C^2X_2C^3X_{51\text{-}52}C^4X_{9\text{-}12}C^5X_{35\text{-}36}C^6X_2C^7X_{14}C^8X_{8\text{-}9}C^9X_8C^{10}$) are also found in dipteran and lepidopteran PMs [12, 15]. Their simplicity implies that simple peritrophins do not contribute greatly to PM structure, and it has been proposed that they may cap the ends of the chitin chains or fill voids not occupied by the more complex peritrophins to protect the chitin scaffold from degradation by endo-chitinases [41] present in the midgut. Indeed, depletion of two simple peritrophins (Pro1 and Pro2) from the *Glossina morsitans morsitans* (tsetse fly) PM does not compromise PM structure [42].

3.2.1.2 Binary Peritrophins

The binary peritrophins also have a simple structure with two CBDs separated by an MD [40], but are classified separately since closely related orthologs are found in several lepidopterans (Fig. 3.1), as well as in some dipterans [12]. Their apparent ubiquity suggests they have a conserved and vital role in PM function. The binary peritrophin MD and its associated carbohydrates contribute more to the total molecular weight of the protein than in any other class of peritrophin. The *M. configurata* binary peritrophin McIIM2 is resistant to degradation by the baculovirus-associated metalloprotease enhancin [43]. This enzyme aids in penetration of the PM by virions by targeting partially glycosylated, but not fully glycosylated, forms of the complex peritrophins described below [21, 38, 43]. Therefore, binary peritrophins may protect epithelial cells from pathogens by restricting access to them by way of their high levels of *O*-linked glycosylation.

3.2.1.3 Complex Peritrophins

Complex peritrophins are a type of IIM and contain multiple CBDs and MDs and are found in both dipteran and lepidopteran PMs. While the number and arrangement of CBDs and MDs differ among the lepidopteran complex peritrophins, they all possess a unique modified PAD at the carboxy-terminus containing two additional conserved cysteines (Fig. 3.1) [15, 21, 30, 37, 38, 44–46]. It has been speculated that these additional cysteines may be involved in intermolecular interactions

leading to mucin multimerization [11] as found in vertebrate mucins [47]. Spacer elements between the CBDs often contain asparagine residues, many of which are predicted to be *N*-glycosylated. Two GlcNAc residues lie at the core of these *N*-linked glycans which are linked by a β-(1, 4) bond as in chitin. These may mimic chitin and be recognized by CBDs leading to further peritrophin-peritrophin interactions.

Unlike genes encoding repetitive or simple peritrophins, expression of lepidopteran IIM genes is specific to the midgut [44]. Interestingly, IIM synthesis does not appear to be tightly coordinated with PM formation as gene expression and protein synthesis are continuous in feeding and starved larvae, as well as during the molt, including pre-molt larvae where a PM is absent [44]. Similarly, early pharate *Trichoplusia ni* (cabbage looper) larvae lack a PM even though IIM is present in midgut epithelial cells [48].

IIMs have several features that contribute to protection of the PM and the underlying epithelium from the lumen environment and its contents. MDs are regions with elevated levels of threonine and serine residues with a carbohydrate adduct linked to them via *O*-linked glycosylation [49]. In many complex peritrophins, glycosylation associated with the MDs can account for 50–90 % of the protein mass [21, 38, 47]. Proline residues are also regularly interspersed among the threonine-serine residues and cause the region to condense into a coil with the carbohydrate moieties projecting outward, sometimes referred to as a "bottlebrush" structure [47, 50, 51]. Interaction of CBDs with adjacent chitin chains contributes to PM structural integrity, while the high degree of glycosylation associated with the MDs imparts a viscous property to the PM that helps to lubricate the luminal tract and facilitate passage of food through the midgut. In *Tribolium castaneum* (red flour beetle), the complex peritrophin (TcPMP5-B) is required for maintenance of PM integrity and barrier functions as depletion of its mRNA via RNA interference increases PM porosity [52].

The PM is a barrier to ingested pathogens [53], and disruption of the PM increases the efficacy of baculoviruses infecting per os [54, 55]. Indeed, many lepidopteran baculoviruses produce a metalloprotease that specifically degrades complex peritrophins, destabilizing PM structure and permitting access to the midgut epithelium [38, 43, 56, 57]. *Bacillus thuringiensis* produces a similar metalloprotease, Bel, which degrades *Helicoverpa armigera* (cotton bollworm) IIM and increases the toxicity of Cry1A δ-endotoxin, presumably by disrupting PM integrity and allowing the toxin to access more easily receptors residing on the underlying epithelial membrane [58].

The insect lumen is a formidable environment. One of the first functions attributed to the PM was to protect the midgut epithelium from damage by abrasive foodstuffs. Indeed, a silkworm, *Bombyx mori*, specimen that lacked a PM in larval stages exhibited a highly abraded midgut epithelium during feeding [59]. In lepidopteran larvae, the lumen is generally caustic and contains copious amounts of hydrolytic enzymes, including proteases [60, 61]. Lepidopteran IIMs are highly resistant to digestion from endogenous midgut serine proteases [37]. The MDs of IIMs have noticeably reduced levels of charged (arginine and lysine) and large polar (histidine,

leucine, methionine, phenylalanine, tryptophan, and tyrosine) amino acids, which are the cleavage sites for trypsins and chymotrypsins, respectively. The glycosylated moieties projecting outward from MDs into the lumen may also sterically hinder proteases from accessing PM proteins. In support of this notion, *T. ni* IIM is susceptible to degradation by endogenous digestive proteases only upon treatment with *O*-glycosidase [37] or dithiothreitol, a disulfide bond reducing agent [38]. Furthermore, trypsin and chymotrypsin residues in CBD are generally located within the domain's interior and are inaccessible to proteases [62]. Glycine and proline residues are common in the spacer regions separating MDs from CBDs in many IIMs, for example, *Plutella xylostella* (diamondback moth) [45] and *Epiphyas postvittana* (light-brown apple moth) and *H. armigera* IIM [46]. These amino acids allow tightly coiled structures to form that should limit access of proteases to potential cleavage sites. In addition, the preponderance of negatively charged residues in these spacer regions may repel serine proteases and prevent them from interacting with PM proteins [44].

Several IIMs, such as *Spodoptera exigua* (beet armyworm) SeIIM and *H. armigera* HaIIM1 and HaIIM86 (Fig. 3.1), possess domains that are replete with charged amino acids interspersed with cysteine residues. Disulfide bridges between cysteines may configure these regions into compact domains with charged amino acid R-groups available for ionic interactions. Indeed, other cysteine-rich regions in IIM bind heavy metals, such as heme groups, and prevent the accumulation of these toxic metabolites [63].

3.2.1.4 Repetitive Peritrophins

Repetitive peritrophins consist of long concatemers of CBDs (Fig. 3.1). The CBDs tend to be very similar to one another indicating that they arose from recent, rapid duplication events. The largest repetitive peritrophin reported, *M. configurata* McPM1, has 19 tandem CBDs [21]. HaIIM2 from *H. armigera* [30] is classified as a repetitive peritrophin even though many of the CBDs are separated by an MD since closer examination reveals that the MD must have been associated with a CBD prior to the initial duplication event. It also lacks the modified carboxy-terminal PAD common to the complex peritrophins. Despite their large initial size, repetitive peritrophins are found as smaller concatemers in the PM [21, 62]. Arginine and lysine residues are often located immediately downstream of each CBD and are likely cleaved by lumen trypsins to form the smaller products. This is difficult to rationalize, since intact repetitive peritrophins could interact with multiple chitin chains and provide enormous structural support. However, the PM must also be permeable, and selectively so, and it has been demonstrated in a coleopteran system that elimination of specific PM structural proteins via RNA interference alters this characteristic [52]. Clearly, a balance exists between structural integrity and permeability. Given the limited repertoire of PM structural proteins present in lepidopteran PMs, fine coordination of repetitive peritrophin processing by proteolytic enzymes

into a myriad of additional products may provide this flexibility. The expression of repetitive peritrophin genes is downregulated by 20-hydroxyecdysone, which signals entry into the molt and cessation of feeding [64].

3.2.2 Enzymes

The PM separates the midgut cavity into three compartments: the endoperitrophic space (the gut lumen including the food bolus), the ectoperitrophic space (between the PM and the epithelium), and the PM itself. This spatial separation of digestive processes allows larger macromolecules (proteins, complex carbohydrates, lipids, etc.) to be partially digested in the endoperitrophic space. This is followed by the movement of smaller products (peptides, complex sugars, fatty acids) through the PM to the ectoperitrophic space where enzymes, such as amino- and carboxypeptidases [65, 66], glucoamylase [67], trehalase [68], and alkaline phosphatase [69], complete the final stages of digestion. In this simple model, the PM serves only as an inanimate, selectively permeable membrane; however, proteomic studies revealed that a plethora of digestive enzymes are tightly associated with the PM [15, 17, 30, 32, 34, 35]. More likely, the PM serves as a three-dimensional scaffold to which digestive enzymes associate. This may allow for spatial coordination of polymer processing as they are reduced in length and pass through the PM, as well as for retention of digestive enzymes in the gut [7].

3.2.2.1 Chitin Deacetylase

Chitin deacetylases (CDAs) are involved in the release of acetyl groups from chitin to form chitosan, the deacetylated form of chitin. CDAs are present in the PM of several lepidopteran larvae, including *T. ni* [70], *M. configurata* [15, 17], *H. armigera* [30], and *B. mori* [71, 72]. Some CDA genes are exclusively expressed in the midgut, such as *TnPM-P42* from *T. ni* [70] and *McCDA1* and *McCDA2* from *M. configurata* [15, 17], while others have broader expression profiles, for example, *H. armigera HaCDA5a* and *HaCDA5B* are expressed in the midgut, Malpighian tubules, and fat body [73]. There is debate as to the role(s) CDAs play in PM physiology as they are not as tightly associated as the peritrophins and therefore unlikely to provide structural support. *HaCDA5a* is downregulated after virus infection, and a recombinant nucleopolyhedrovirus expressing *HaCDA5a* increases PM permeability and is more infective than the parental virus. This suggests that downregulation of midgut CDAs may reduce susceptibility to baculovirus infection by decreasing PM permeability [73]. Indeed, deacetylation decreases the density of chitin fibrils and alters their structure and orientation [74, 75]. CDA activity is present in the midgut [17], though the presence of chitosan in the PM has not been demonstrated [3, 30, 76]. Deacetylation of PM chitin would not only alter its physical and chemical properties but also affect binding of PM proteins and therefore PM integrity and permeability.

3.2.2.2 Chitinase

Endo-chitinases are associated with the PM of lepidopteran larvae [77] and dipteran adults [32]. Their expression peaks during the molt, suggesting that they are primarily involved in turnover of the PM during the molt [77], though a basal level of chitinase expression is also present in feeding *M. configurata* [78] and *H. armigera* [79] larvae, as well as in some beetles [80, 81], suggesting that these enzymes may play a role in subtle trimming or modifying the PM, rather than simple degradation. Chitinase gene expression and activity also increase upon feeding in mosquitoes [82–84]. Furthermore, ingestion of a chitinase inhibitor leads to the formation of an atypical, thicker PM, confirming that a basal level of chitinase activity is required during feeding for proper PM formation [83]. Larvae fed double-stranded RNA targeted against *Ostrinia nubilalis* (European corn borer) chitinase (*OnCht*) mRNA have increased levels of chitin in their PM but also exhibit lower larval body weight than control larvae [85], providing further evidence that chitinase activity affects PM chitin content and its physiological properties.

3.2.2.3 Proteases

The pH of the lepidopteran larval digestive tract is highly basic, up to pH 11.0, and this alone dictates that the digestive protease composition must be almost exclusively serine proteases [86, 87]. Some of the first PM proteins discovered were serine proteases [88, 89], and subsequent proteomic analyses revealed that the lepidopteran PM hosts a plethora of trypsins, chymotrypsins, and elastases [15, 30, 34, 35, 44]. The strength of association of serine proteases with the PM is somewhat debatable as they may simply have become entrapped while in transit to the lumen; however, an equally probable scenario is that the PM anchors proteases and other digestive enzymes to reduce enzyme loss and greatly improve digestive efficiency [90]. Many of the serine proteases found in the lepidopteran larval gut and/or PM lack one or more of the critical serine, histidine, and aspartic acid residues required for catalytic activity and are likely to be inactive [30, 60, 61]. The abundance of non-catalytic forms suggests that they contribute something to insect digestive biochemistry, possibly sequestering protease inhibitors in the diet.

An astacin-like zinc metalloprotease, McAST, was identified in the *M. configurata* PM [15]. Astacins and astacin-like enzymes are extracellular proteases and may be membrane bound or secreted. They are ubiquitous among higher eukaryotes and have been implicated in processes ranging from development to digestion [91]. *McAST* was exclusively expressed in the midgut, as was the astacin-encoding gene from *G. m. morsitans* [92]. These enzymes have been implicated in digestion [92] and protection of the insect from *B. thuringiensis* δ-endotoxins [93].

The short peptides released by endopeptidases are digested into component amino acids in the ectoperitrophic space and on the microvillar membrane by soluble and membrane-bound amino- and carboxy-exopeptidases [94]. Both types of enzymes were found in the PM of *H. armigera* [30], *B. mori* [34, 35, 95], and

M. configurata [15] larvae where they may hydrolyze polypeptides that are too large to pass through the PM. Aminopeptidases are receptors for *B. thuringiensis* δ-endotoxins [96] which are attached to the epithelial membrane by a glycosylphosphatidylinositol (GPI) anchor [97]. Enzymes released from the epithelium [87] may associate with the PM and intercept toxins as they move through the PM. Indeed, binding of *B. thuringiensis* δ-endotoxins by the PM followed by excretion in the frass contributes to the resistance of some lepidopteran species to these agents [98].

3.2.2.4 Lipases

Insect lipids are best known as a stored source of energy in the fat body which may be used for embryogenesis, reproduction, metamorphosis, long-range flight and during starvation. Insect intestinal lipases (IILs) and related esterases digest dietary fats in the midgut. While the activity of lipases is restricted to hydrophobic-hydrophilic interfaces [99], they are also major components of the lepidopteran larval PM [15, 17, 30]. Similar to midgut serine proteases, many PM-associated lipases lack crucial active site residues and are likely to be inactive [17, 22]. *M. configurata* genes encoding active McIIL1 and McIIL2 are expressed during feeding, while expression of the gene encoding the inactive McIIL3 peaks during the molt [17]. This implies that the active and inactive forms may have very different roles during insect development.

How hydrolytic enzymes associate with the PM is unclear, but lepidopteran PM-associated lipases have a conserved register of cysteine residues ($CX_4CX_{22}CX_{10}C$) near their carboxy-termini [15, 17]. The motif is highly conserved among pancreatic lipases found in the lepidopteran midgut, but is not present in pancreatic lipases from other tissues, such as accessory glands and yolk, or human or rat pancreas. While this cysteine register does not conform to any known CBD in peritrophins or cuticle proteins [100], it is possible that it is a new type of binding domain that allows insect intestinal lipases to associate with the PM.

3.2.2.5 Other Enzymes

β-1,3-glycosidic bonds in β-1,3-glucans are hydrolyzed by β-1,3-glucanases (β1,3GLU). *H. armigera Haβ1,3GLU* and *Spodoptera frugiperda* (fall armyworm) *β1,3GLU* are expressed in the midgut [101], and Haβ1,3GLU [30] and *M. configurata* β1,3GLU [15] are associated with the PM. Midgut β1,3GLU is involved in digestion as well as protection from pathogens, as β-1,3-glucans are structural components of fungi that may be ingested with the diet [102].

The α-(1,4) glycosidic bonds in starch and glycogen are hydrolyzed by α-amylases yielding glucose and maltose. Midgut α-amylases are present in the lepidopteran larval midgut lumen [31, 33] and the PM [15, 30, 89, 90]. α-Amylases

are essential for insects feeding on starch-rich diets, such as stored products or roots. Most α-amylases require Ca^{2+} and Cl^- for activity; however, the Mediterranean flour moth, *Ephestia kuehniella*, alpha-amylase (EkAMY) has a mutation in the Cl^- binding site, and its activity is Cl^- independent [103]. This mutation is common in lepidopteran α-amylases and may be an adaptation to the alkaline midgut environment.

Alkaline phosphatases (ALPs) catalyze the release of phosphate groups from organic molecules. Midgut ALPs may be soluble (s-ALP) or attached to the membrane (m-ALP) of columnar or goblet cells by a GPI anchor [104, 105]. m-ALPs were associated with the *H. armigera* [30] and *M. configurata* [15] PM suggesting that the GPI anchor is cleaved and the ALP released. Interestingly, s-ALPs were not found in the tobacco budworm, *Heliothis virescens*, midgut [106]. The role of ALP in PM function can only be speculated. Removal of phosphate groups from proteins may facilitate passage through the ionic PM. *B. thuringiensis* δ-endotoxins also interact with *N*-linked glycans on ALPs [107], and PM-associated ALPs may intercept these toxins as proposed above for aminopeptidases.

A nonspecific DNA/RNA endonuclease was identified in the PM of *M. configurata* [15] and *B. mori* [35]. The *M. configurata* PM also contains a pantetheinase [15] similar to that in the *S. frugiperda* midgut [29].

3.2.3 Other Proteins

3.2.3.1 Lipocalin

Lipocalins are a diverse group of proteins having a β-barrel domain with affinity for small hydrophobic ligands [108]. Polycalins (polymeric lipocalins) are present in the midgut [31, 109] and PM [15, 30] of lepidopteran larvae. Midgut polycalins have been implicated in binding of plant pigments [110] and *B. thuringiensis* δ-endotoxins [111]. Lipocalins aggregate lipophilic allelochemicals, such as digitoxin, to form micelles in the gut that are eliminated by PM-mediated extractive ultrafiltration [112]. Differential splicing of exons can create hybrid lipocalins which increase the diversity of ligands that can be recognized [110].

3.2.3.2 Serpin

Serpins are irreversible inhibitors of serine proteases. Through differential splicing, the lepidopteran *Serpin1* gene encodes as many as 9–12 serpin variants each possessing a different inhibitory loop situated on a common scaffold [113–115]. Interestingly, only the *M. configurata* McSerpin1A variant is found in the midgut where it is associated with the PM [15, 116], though its role is unknown.

3.2.3.3 Lectins

Lectins are carbohydrate-binding proteins which are associated with the PM [117] where they may interact with GlcNAc in the chitin network or with sugar moieties on *N*- and *O*-linked oligosaccharides on PM proteins. A C-type lectin, McCLECT, is associated with the PM of *M. configurata* [15]. McCLECT and other lepidopteran CLECTs [118] have an EPD motif in the carbohydrate recognition domain indicating that they bind glucose or mannose. McCLECT may therefore associate with the PM by binding mannose moieties on the *O*-linked glycans of IIMs. *McCLECT* expression is not exclusive to the midgut, which is similar to other insect CLECT genes [28, 119]. CLECTs are known to participate in innate defense responses [120], and PM-associated lectins protect the tsetse fly gut from trypanosome infections [121].

3.2.3.4 REPAT (Response to Pathogen) Proteins

Seven different REPAT orthologs were identified in the *M. configurata* PM [15]. The *M. configurata* REPAT genes are expressed only in the midgut; however, *S. exigua* REPAT genes are also expressed in the hindgut [122] and those from *H. armigera* in the fat body [123, 124]. The induction of REPAT gene expression in response to pathogen challenge and the reduced virulence of a baculovirus expressing SeREPAT1 suggest that they play a role in defense against pathogens [122].

3.2.3.5 Cysteine-Rich Proteins

Two PM proteins detected in the head, hemolymph, and integument of *Loxostege sticticalis* larvae, Lsti99 and Lsti201 [125], share homology with PM proteins from the *M. configurata* PM [15]. These proteins have conserved cysteine registers at the amino- ($C^1X_2C^2X_{19\text{-}20}C^3X_{34\text{-}41}C^4$) and the carboxy-terminus ($C^1X_{40\text{-}43}C^2X_{19}C^3X_{17}C^4X_{16}C^5$), but they do not conform to known CBDs. The function of these proteins is unknown.

3.3 Molecular Model of the Peritrophic Matrix

The molecular model of the lepidopteran PM has evolved as genomic and proteomic studies uncovered new PM-associated proteins [11, 14, 15, 21]. The most current model includes what is likely the complete set of lepidopteran PM structural proteins, as well as enzymes involved in chitin metabolism (Fig. 3.2). Peritrophins containing multiple CBDs, such as McPM1 [21] and *T. ni* chitin-binding proteins

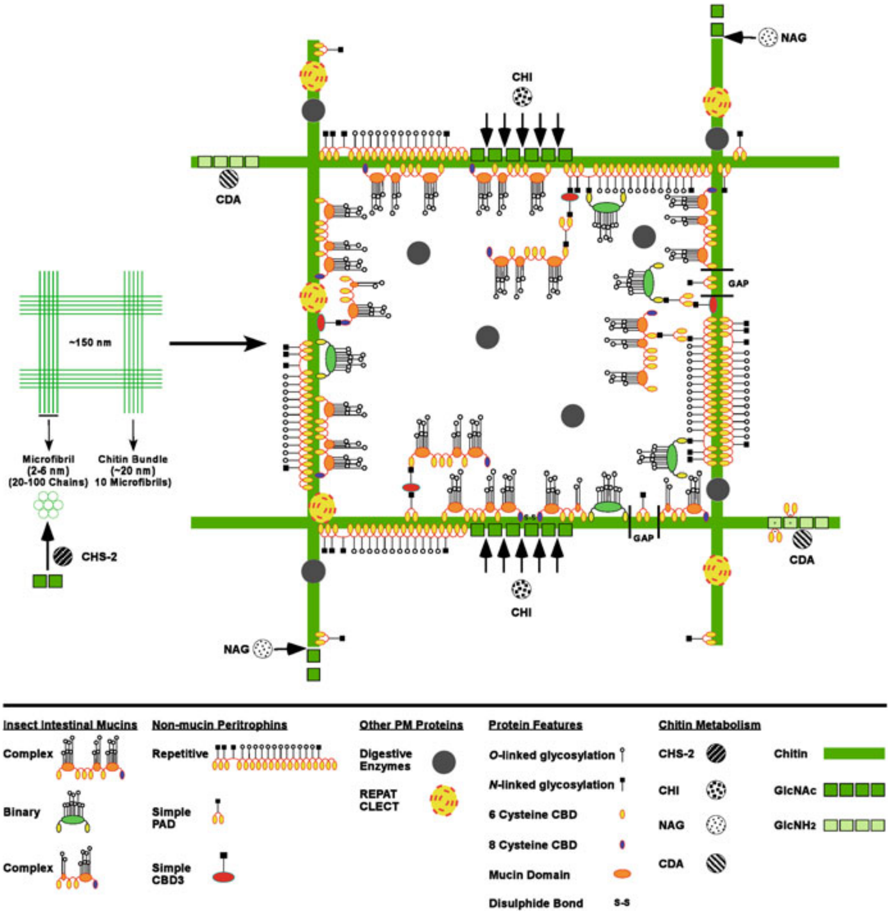


Fig. 3.2 Model of lepidopteran peritrophic matrix architecture. *CBD* chitin-binding domain, *CBD3* Type III chitin-binding domain protein, *CDA* chitin deacetylase, *CHI* endo-chitinase, *CHS-2* chitin synthase 2, *CLECT* C-type lectin, *GlcNAc* *N*-acetylglucosamine/chitin, *GlcNH2* deacetylated chitin/chitosan, *NAG* *N*-acetylglucosaminidase, *PAD* peritrophin-A domain, *PM* peritrophic matrix, *REPAT* response to pathogen

1–2 (TnCBP1–2) [62], once again feature prominently as they likely hold the chitin fibrils and bundles together. Peritrophin multimerization, either through intermolecular disulfide bridges or interaction of CBDs with *N*-linked glycan cores, increases the spatial complexity and structural stability of the PM. Simple peritrophins containing only one or two CBDs cap the ends of the chitin fibers or occupy spaces remaining after assembly of microfibrils. Included in the model are enzymes involved in chitin turnover (chitinases) and modification (CDA) as they affect PM architecture.

3.4 Update on Lepidopteran Peritrophic Matrix Formation

Richards and Richards [126] outlined the mechanisms employed by insects of different taxa to synthesize a PM. The PM can be synthesized along the entire length of the midgut epithelium (Type I) or secreted from the cardia (Type II), a specialized organ near the foregut-midgut junction [3, 127]. The Type II mode of PM synthesis appears to be restricted to dipteran larvae. The Type I mode is found in most lepidopteran larvae, with the possible exception of early pharate larvae, as well as in female hematophagous dipteran adults where it is formed in response to feeding [3, 128]. A Type I PM is also found in Coleoptera, Dictyoptera, Ephemeroptera, Hymenoptera, Odonata, Orthoptera, and Phasmida [3, 6]. Although they subsist mainly on fluids, lepidopteran adults also have a PM [118]; however, its structure and composition has not been well studied as this stage does not directly contribute to economic losses. The morphology of the PM from molting lepidopteran larvae also differs from that found in feeding larvae [44]. The PM from feeding *M. configurata* larvae is thinner and composed of approximately 5–10 layers. As larvae enter the molt and feeding ceases, the PM is degraded; however, PM synthesis resumes shortly thereafter, and by mid-molt the lumen is filled with a chitinaceaous, multi-laminate PM-like material. This is eventually expelled and replaced by a feeding-type PM in early post-molt larvae [78]. The physiological role of the molting PM is not known, but it may serve to protect the midgut epithelium from hydrolytic enzymes which persist in the midgut during the molt [44] or provide structural support when the gut is devoid of food. The morphology of the PM also differs along the length of the midgut [48, 129].

In dipteran larvae, the PM is secreted from cardial invaginations to form a multi-laminate membrane [117]; however, PM synthesis in lepidopteran larvae is somewhat more complicated and appears to mature as it passes along the midgut. In *T. ni*, the PM is produced along the entire midgut epithelium beginning with the secretion of an amorphous material that matures into a cross-linked fibrous network [130]. The *H. virescens* PM is secreted from an annular ring of cells at the junction between the foregut and midgut, somewhat analogous to that of the cardia [131]. In *O. nubilalis*, a single layer PM is formed in the anterior midgut, which becomes progressively thicker as new layers are applied along the length of the midgut [129].

PM structural proteins and enzymes are delivered to the lumen via microapocrine vesicles that emerge from the midgut microvilli [132]. Gene expression and proteomic studies have provided additional clarity as to how the lepidopteran PM forms and matures. Lepidopteran PM appears to form via a hybrid model that is something between the traditional definition of the Type I and Type II modes of PM formation [133]. In this model, the main structural PM proteins are incorporated into the PM in the anterior region, such as the repetitive peritrophins SfPER-33 K from *S. frugiperda* [90], polyCBD from *H. armigera* [30], and McPM1 from *M. configurata* [78], as well as some of the IIMs, for example, HaIIM4 from *H. armigera* [30] and McIIM1 and McIIM2 from *M. configurata* [78]. CDA is also incorporated in the anterior region [30], but does not remain associated with the PM

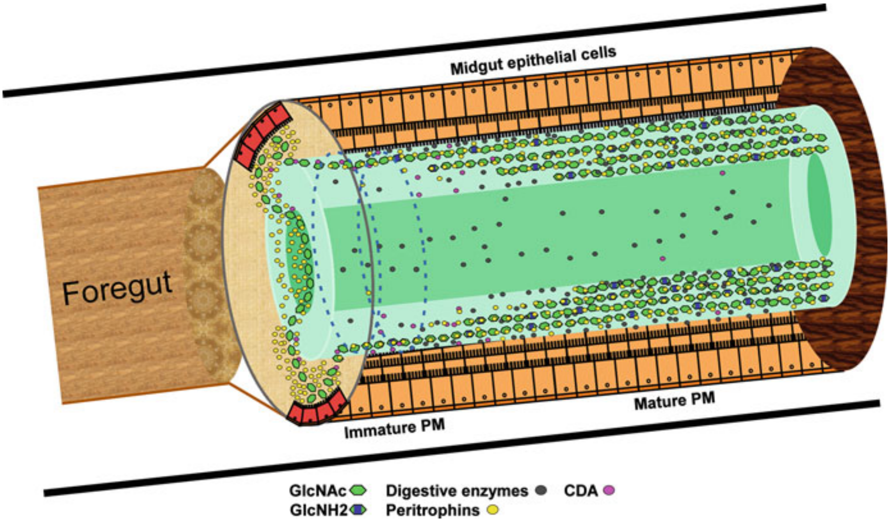


Fig. 3.3 Model for lepidopteran peritrophic matrix formation. *CDA* chitin deacetylase, *GlcNAc* *N*-acetylglucosamine/chitin, *GlcNH2* deacetylated chitin/chitosan

as it migrates toward the hindgut [78]. Other IIMs are introduced later in the middle and posterior midgut [48].

Studies on PM chitin synthesis in lepidopterans, including the jute hairy caterpillar, *Diacrisia obliqua* [134], *H. virescens* [131], *O. nubilalis* [129], *T. ni* [48], and the tobacco hornworm, *Manduca sexta* [133], support this model. These studies revealed that chitin synthase 2 (*CHS-2*) gene expression is highest in the anterior midgut and that this region is the major site of PM chitin production [135–137]. As noted above, genes encoding many PM-associated proteins are expressed predominantly in the anterior midgut of feeding larvae. Collectively, these observations support the notion that the anterior midgut is the site of initial PM synthesis and that maturation ensues further along the midgut with the deposition of additional PM proteins and chitin layers (Fig. 3.3).

3.5 Future Directions

It is very likely that the entire complement of PM-associated proteins has been discovered. If this statement holds true, the multitude and diversity of functions attributed to the PM is astonishing given its seemingly simple structural complexity. Future studies will need to dissect the role of individual PM components. This has been possible in dipteran [53] and coleopteran [52] systems where functional genomics tools are well established. Techniques to apply RNA interference in lepidopteran systems are under development [138, 139], but delivery of double-stranded RNA has proven useful in targeting several genes encoding

midgut-specific proteins, such as digestive enzymes [140–142], PM structural proteins [143], chitin synthase [144], chitin-modifying enzymes [85, 143], toxin receptors [145], and detoxification enzymes [146]. Practical methods for per os delivery of dsRNA including bacteria [147] or transgenic plants [148–150] are leading to new, highly specific strategies for the control of insect pests.

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Chapter 4
Cold Adaptation Responses in Insects and Other Arthropods: An "Omics" Approach

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Abstract In this chapter, we review recent genomic, proteomic, and metabolomic studies that link several gene and protein products involved in cold adaptation in insects and other arthropods to energy metabolism and cellular protection mechanisms. Organisms have evolved various mechanisms for survival at subfreezing temperatures. In general, cold hardy invertebrates utilize four main strategies to survive cold temperatures: (1) freeze tolerance, (2) freeze avoidance, (3) cryoprotective dehydration, and (4) vitrification. In addition, many insects in temperate regions overwinter in an arrested developmental state known as diapause, during which they are cold hardy. Major alterations occur during winter diapause, with respect to both total metabolic flux and the relative activities of different metabolic pathways. In these organisms, one such metabolic adaptation to unfavorably cold environmental conditions is the synthesis of cryoprotectants/anhydroprotectants. The metabolic changes and metabolic paths involved in cold adaptation suggest involvement of specific enzymes and key regulatory proteins. These mechanisms of cold adaptation require precise scheduling of the expression of specific genes. Thus, we discuss here the evidence researchers have recently begun to gather supporting a relationship between the genes and proteins of the cold adaptation response and mechanisms of cellular protection and energy metabolism using an "omics" approach.

Abbreviations

ADS	Antioxidant defense system
AFP	Antifreeze protein
CDMC	Canadian *Drosophila* Microarray Centre

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CLT	Constant low temperature
CS	Cold shock
2DE	Two-dimensional gel electrophoresis
2DE-DIGE	Two-dimensional fluorescence difference gel electrophoresis
EST	Expressed sequence tag
GC–MS	Gas chromatography–mass spectrometry
GlyP	Glycogen phosphorylase
GO	Gene Ontology
HSP	Heat shock protein
INP	Ice-nucleating protein
iTRAQ	Isobaric tag for relative and absolute quantitation
LC–MS	Liquid chromatography–mass spectrometry
LC-MS/MS	Liquid chromatography–tandem mass spectrometry
NCBI	National Center for Biotechnology Information
NMR	Nuclear magnetic resonance
Q-PCR	Quantitative polymerase chain reaction
RCH	Rapid cold hardening
SSH	Suppression subtractive hybridization

4.1 Introduction

Historically, new insights into molecular physiology and signal transduction have closely followed the development of improved scientific methods and instrumentation, leading to more profound and better understanding of the regulation of life processes. Because of these advances in technology, we can now discuss more precisely the genes, proteins, and molecular species involved in governing function, time coordination, and myriad interactions between organisms and their environment. This complex web of interactions appears to be amplifying continually, including more and more molecular modifications, relationships, and possible metabolic routes. Thus, to begin to describe fully even just a single life process requires broad experimentation and effort to unravel the key components and their relationships with other processes. This description is often complicated by the need to consider more than one species, genome, proteome, and metabolome, in order to help identify key molecular players or pathways. To overcome these problems, new scientific tools and bioinformatics techniques have been developed which provide a "wide-angle" view, based on collection of enormous amounts of data concerning gene expression, protein products, posttranslational modifications, and intra- and intermolecular interactions associated with a particular process. Collectively, these methods are referred to as "omics" approaches, including genomics, transcriptomics, proteomics, and metabolomics. Further, analysis of complex processes requires an integrated "omics," focusing on answers to questions such as: What are

the key sequences, pathways, or molecular players involved in such a diversity of life strategies? How many of them are involved? And (importantly) which processes are worth the effort and cost of such explorative techniques? Recent attempts to find the relationship between mechanisms of cold adaptation in cold hardy insects and other arthropods, including energy metabolism and cellular protection, is a perfect example of where nearly all biological scientific disciplines are involved. Resistance to cold exposure in arthropods is not just a fight against cold, it is to fight to live and progress. Over a million insect species are spread out all over the world and thriving in virtually all possible climates, including polar regions. In fact, it can be said that insects are great masters of survival in cold extremes, with adaptation strategies combining survival and larval development.

In general, cold hardy invertebrates utilize four main strategies to survive cold temperatures: (1) freeze tolerance, (2) freeze avoidance, (3) cryoprotective dehydration, and (4) vitrification (Fig. 4.1). In addition, even in more temperate regions, many insects overwinter in a state of arrested development called diapause, during which they display significant cold hardiness. Major physiological alterations occur during this winter diapause, with respect to both total metabolic flux and the relative activities of different metabolic pathways. In these insects, one such metabolic adaptation to unfavorably cold environmental conditions is body water redistribution along with the synthesis of cryoprotectants or anhydroprotectants (e.g., polyols, sugars, and amino acids), the presence of which is now considered to be a hallmark of cold hardiness [1]. The metabolic changes and metabolic pathways involved in cold adaptation suggest the involvement of specific enzymes and regulatory proteins, requiring precise scheduling of the expression of specific genes [2]. Changes in lipid composition are essential for membrane fluidity and its function [3–5]. Such metabolic changes cause subsequent production of reactive oxygen species, which

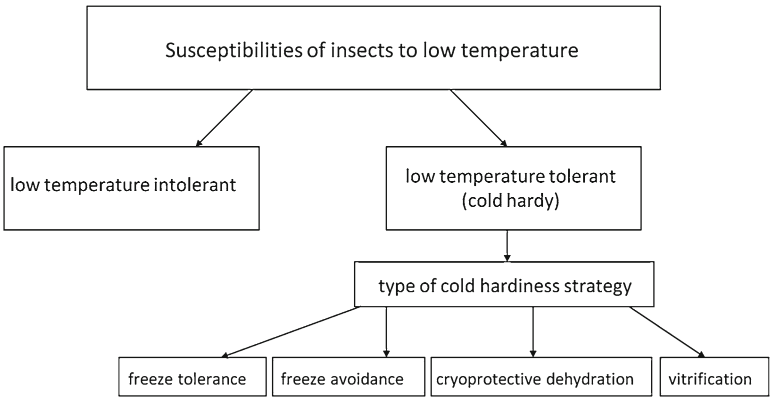


Fig. 4.1 Scheme showing susceptibilities of insects to low temperature and the main strategies of insect cold hardiness

provokes physiological responses including the antioxidative defense system [6–8]. In fact, the apparent complexity of cold adaptation processes and the possible insight(s) that could arise from its investigation seem to require an integrated "omics" approach.

4.2 Genomics and Transcriptomics Research on Insect Cold Hardiness

Technological advances in the late 1990s have enabled whole-genome sequencing to become economically feasible, marking the beginning of the so-called genomic revolution and ushering in the post-genomic era. In 2000, the genome of the fruit fly, *Drosophila melanogaster*, a model organism in genetics, molecular, and developmental biology for over a century, was one of the first eukaryotic genomes to be sequenced [9]. Subsequently, a large number of *Drosophila* species have been sequenced in order to provide a clearer picture of the *D. melanogaster* genome and to provide data for functional, phylogenetic, and population analyses [10, 11]. Since then, the number of species whose genome has been completely sequenced has constantly grown, and the pace of these efforts has been accelerated recently with the development of next-generation sequencing platforms. According to the National Center for Biotechnology Information (NCBI) data (as of June 2014) (http://www.ncbi.nlm.nih.gov), the database of genomic projects contains information on 98 insect species: some with a fully sequenced genome, some with a draft sequence, and some still in the process of sequencing. In addition to these, sequenced genomes exist which are not available in public databases. In 2011, the i5k initiative was started, with the goal of sequencing 5000 insect and related arthropod species over the next five years [12]. Frequently, the choice of species whose genome will be sequenced is driven by its economic importance or role as vector in the transmission of serious human diseases. Among the several dozen completely sequenced genomes, there are four species that may be important for genomic analysis of low-temperature resistance [2]: *Culex quinquefasciatus* (adult reproductive diapause in close relative *C. pipiens*) [13], *Bombyx mori* (obligate, cold hardy, embryonic diapause phases) [14, 15], *Acyrthosiphon pisum* (egg diapause, high-latitude distribution) [16], and *Tribolium castaneum* (overwinters all stages, rapid cold hardening; RCH) [17]. In addition, there is a significantly larger number of species with available expressed sequence tag (EST) information. EST sequences have had a significant impact on the analysis of transcriptomic data, because they provide a platform for printing custom cDNA microarrays, and for the molecular characterization of clones, since commercial microarrays are often not available for the model organisms used in cold tolerance research. The contribution of "omics" research on insect cold hardiness has been reviewed in Denlinger and Lee [2] and Storey and Storey [18]. A brief overview of low-temperature responsive proteins/gene products across

species (identified by bioinformatics-based approaches) is given by Carrasco et al. [19], including a database containing 2030 entries across 34 different species, with two collembola species (*Megaphorura arctica*, *Cryptopygus antarcticus*) and five insect species (*Aphidius colemani*, *C. pipens*, *D. melanogaster*, *D. virilis*, *Sarcophaga crassipalpis*).

4.2.1 Transcriptomic Analyses of **Drosophila** *and Other Insect Species*

Given the central role of *D. melanogaster* as a model organism in the development of genomics – Affymetrix launched its first *D. melanogaster* array in 2000 – it is not unexpected that most "omics" technologies have been applied to the study of this species. Although *D. melanogaster* is not freeze tolerant and is not an ideal model system for the study of mechanisms of low-temperature survival, a lot of work has been done regarding thermal adaptation in this species, where the differences between slow seasonal and rapid cold hardening (RCH) could be investigated. These data are extremely important because they form a basis for comparison of genes and EST sequences isolated from species known to be better cold survivalists. Like *D. melanogaster*, other members of the Drosophilidae family are generally cold sensitive [20], with the exception of *Chymomyza costata* larvae which have been shown to be freeze tolerant [21].

The first transcriptomic experiments on cold acclimation in *D. melanogaster* were conducted using suppression subtractive hybridization (SSH) [22, 23], suggesting that a gene homologous to senescence marker protein 30 (*SMP30* or *Dca*) is upregulated after acclimation to 15 °C. Increased expression of this gene was also observed in individuals reared from the egg stage at 15 °C versus 25 °C. Subsequently, the *Frost* (*Fst*) gene was shown to be strongly upregulated during recovery from a period of cold shock at 0 °C. The first microarray analysis of *D. melanogaster* cold tolerance was performed by Qin et al. [24]; initially, this work was done using commercially available 7000 sequence microarrays and later with 12,000 sequence arrays when they became available from the Canadian *Drosophila* Microarray Centre (CDMC) (http://www.utm.utoronto.ca/~w3flyma/overview.htm). Transcription was analyzed following 2 hr exposure of embryos to 0 °C followed by recovery at 25 °C versus those kept at a constant 25 °C. These experiments indicated the importance of *Frost*, heat shock proteins, *HSP23*, *HSP26*, and *HSP83* and certain membrane-associated proteins in the biological response to cold shock. In contrast, another *D. melanogaster* microarray study conducted over ten generations [25] failed to reveal any significant changes in expression patterns associated with increased resistance to cold stress. On the other hand, Telonis-Scott et al. [26] reported variations in expression pattern associated with chill coma stress, which is induced by prolonged exposure to critically low temperatures and is characterized

by complete movement cessation. Prolonged exposure to such temperatures results in chill-induced injuries. However, there are some fundamental differences between these two studies, both with respect to selection regime (e.g., in Telonis-Scott, females were selected without acclimation) and microarray experiments that could account for these different results. In Telonis-Scott et al., 94 differentially expressed genes were identified by microarray associated with a number of functions and pathways, including proteolysis, electron transport, and the immune response. Illustrating the power of these methods, only a few of these 94 genes have been previously associated with stress resistance, and there was no apparent overlap with genes previously proposed as candidates for cold resistance. Zhang et al. [27] compared the effects of short, single prolonged, and repeated exposure to cold (−0.5 °C) using oligonucleotide microarrays from the CDMC with 72,000 probes, corresponding to 15,473 *Drosophila* genes. They identified 20 genes upregulated in response to a single short exposure to cold, 69 genes in response to a single prolonged exposure, and 76 genes upregulated in response to multiple exposures, with a small degree of overlap. Perhaps surprisingly, only three genes (*Turandot A*, *Hephaestus*, and *CG11374*) were found to be upregulated in all three treatments. These three genes are not functionally related: *Turandont A* encodes a humoral immune factor, whereas CG11374 encodes a protein with galactose binding and urate transmembrane transporter activity and *Hephaestus* encodes an mRNA-binding protein involved in notch signaling and morphogenesis. Their exact roles remain to be explored. Interestingly, many of the genes uncovered had been previously implicated in muscle structure and function, the immune response, stress response, carbohydrate metabolism, and egg production.

The first cross-species microarray study of cold tolerance using the *D. melanogaster* chip was conducted on the freeze avoiding gall moth species, *Epiblema scudderiana* [28]. The gall moth overwinters as a final instar caterpillar in elliptical galls, and key metabolic adaptations have been proposed to support its subzero survival (near −40 °C), including accumulation of high concentrations of glycerol [29]. Heterologous screening identified altered expression of a large number of putative transcripts in response to exposure to freezing temperatures (−20 °C), including significant upregulation of six plasma membrane transporters. In a particularly ambitious study using the temperate *Drosophila* species, *D. subobscura*, a whole-genome transcriptional response was subjected to cross-species microarray analyses following three years of thermal adaptation [30], using a CDMC microarray complete with 10,500 *D. melanogaster* and 7 *D. subobscura* genes. A total of 306 cDNA clones were observed to be differentially expressed between the two furthest extremes of the designed thermal selection regime (13 °C versus 22 °C). These results indicated the importance of genes involved in carbohydrate and nucleic acid metabolism, as well as regulation of transcription. Moreover, supporting the validity of the method, four genes previously reported to be important in thermal adaptations (*HSP26*, *HSP68*, *Fst*, and *Treh*) were identified.

Combined transcriptomic and metabolomic approaches were used to examine rapid cold hardening and cold shock recovery in the flesh fly, *S. bullata* [31]. While RCH had very minor effects on gene expression, recovery from cold shock was associated with altered gene expression (specifically, differential expression of approximately 1400 ESTs, including a number of heat shock proteins, cytoskeletal components, and genes implicated in several signal transduction pathways) and metabolism, including a dramatic increase in amino acid synthesis, gluconeogenesis, and the synthesis of cryoprotective polyols. Similarly, in a study of the effects of RCH and cold acclimatization on chill coma recovery times in *D. montana* and *D. virilis* analyzed at the transcriptome level by microarray [32], cold acclimatization was found to clearly decrease chill coma recovery times in both species, whereas rapid cold hardening did not appear to have any significant effect. Of the 219 genes studied, two showed rather consistent expression changes: *hsr-omega*, which was upregulated, and *Eip71CD*, which was downregulated in nearly all of the cold treatments. The cold-responsive genes in *D. montana* and *D. virilis* were mainly linked with heat shock response, circadian clock, and metabolism.

The first microarray study to look at differential expression of miRNAs in a freeze-tolerant insect was performed on the goldenrod gallfly larvae, *Eurosta solidaginis* [33], where the role of miRNA in global regulation of translation was analyzed. Expression of miRNA was found to be altered in frozen (−15 °C) versus control (5 °C) larvae. Specifically, levels of miR-11, miR-276, miR-71, miR-3742, miR-277-3p, miR-2543b, and miR-34 were significantly reduced in frozen larvae, whereas miR-284, miR-3791-5p, and miR-92c-3p increased. In this study, TargetScanFly 6.0 software was used for microRNA target prediction. Changes in two miRNAs, miR-277-3p and miR-284, suggested potential regulation of transcripts involved in translation and the Krebs cycle.

4.2.2 Diapause – Transcriptomic Experiments

For many insects inhabiting temperate regions, diapause is associated with winter cold hardening. Using SSH, Robich et al. [34] identified 40 differentially expressed genes associated with diapause in overwintering female mosquitoes (*C. pipiens*). Northern blot hybridizations further confirmed expression of 32 of the SSH clones. Summarizing their results in brief: six genes were found upregulated specifically in early diapause, 17 upregulated in late diapause, two upregulated throughout diapause, two downregulated throughout diapause, and five genes had unchanged expression during diapause. These genes or their products fall into eight distinct functional groups: stress response, metabolism, food utilization, regulatory functions, cytoskeletal components, transposable elements, ribosomal components, and genes with unknown functions.

Rinehart et al. [35] conducted gene expression experiments on the flesh fly, *S. crassipalpis* (which is capable of cold hardy diapauses), following cold acclimation using the SSH technique. The focus of this study was on the role of heat shock proteins. Diapause periods were associated with upregulation of two members of the HSP70 family, one member of the HSP60 family (TCP-1), at least four members of the small HSP family, and a small HSP pseudogene. Expression of *Hsc70* was uninfluenced by the diapauses, and *HSP90* was downregulated. Similarly, in the same species, Rinehart et al. [36] used SSH to characterize the diapause transcriptome. Of 97 unique clones, 17 were observed to be upregulated in the diapause periods, while one was downregulated and 12 others were unaffected. Diapause-upregulated genes included neuropeptides, heavy metal responsive genes, heat shock proteins, as well as structural genes, regulatory elements, and several genes of unknown function. The first large-scale EST project for *S. crassipalpis* was conducted by Hahn et al. [37], using massively parallel pyrosequencing on the Roche 454-FLX platform. Here, 207,110 ESTs were obtained, encompassing approximately 9,000 independently identified transcripts. These EST sequences were subsequently applied by Ragland et al. [38] to construct microarrays capable of comparing gene expression between diapausing and non-diapausing flesh fly pupae and to investigate the changes associated with several stages of pupal diapause. These studies revealed enhanced expression of genes associated with glycolysis and gluconeogenesis, strongly suggesting a reduced reliance on aerobic metabolism in the diapause state [18]. Further, the authors revisited published datasets to compare dormancy regulation among pupal diapause in the flesh fly, adult reproductive diapause of the fruit fly, *D. melanogaster*, and the larval dauer stage of the nematode, *Caenorhabditis elegans*.

Drosophila montana undergoes a reproductive diapause that enables adult flies to survive the harsh winter conditions often found in high latitudes and altitudes. Kankare et al. [39] created a custom microarray for *D. montana* with 101 genes and detected two genes, *Dca* and *cpo*, whose role in photoperiodic diapause in this species is worthy of studying in more detail.

4.2.3 Polar Species – Transcriptomics Experiments

Species that inhabit polar regions somehow manage to survive the extreme polar winters, where temperatures often reach −20 °C, making them natural model organisms to elucidate the mechanisms that enable them to survive under these conditions. The ability of the freeze avoiding Antarctic collembola, *C. antarcticus*, to survive at ultra-low temperatures has been well described at the physiological level [40]. However, the molecular mechanisms underlying this phenomenon remain poorly understood. To begin to address this knowledge gap, two small custom microarrays (672 and 5400 clones) were constructed for this species, based on 3430 EST sequences currently deposited in the NCBI EST database [41, 42]. Both

experiments support a clear role for molting in conferring cold tolerance to *C. antarcticus* [43].

In contrast, the Arctic collembola, *M. arctica*, survive subfreezing temperatures by losing nearly all of their body water in a process known as cryoprotective dehydration [44–47]. For this species, 16,379 EST sequences were generated [48], obtained from both dehydrated and rehydrated populations. Approximately 40 % of these were annotated against the Swiss-Prot and TrEMBL databases and further analyzed using Gene Ontology (GO) annotation. After construction of the EST libraries, microarrays containing 6912 clones were printed [49]. Results strongly suggest that the dominant processes associated with cryoprotective dehydration include mobilization of trehalose and protection of cellular systems via expression of small heat shock proteins, coupled with tissue and cellular remodeling. Supporting this, major genes identified during recovery are known to be involved in cell division, initiation of protein translation and energy production, as well as tissue repair processes. Quantitative polymerase chain reaction (Q-PCR) of selected candidate genes has also contributed to our understanding, with glutathione S-transferase identified as a major antioxidant enzyme involved in protecting cells during cryoprotective dehydration, along with a number of protein kinase signaling molecules involved in recovery. However, further investigation is required, and aquaporins, desaturases, and novel membrane proteins were identified in this study as promising candidates for future analyses.

4.3 Proteomic Studies of Cold Hardiness in Insects and Other Arthropods

Despite the complex and simultaneous changes in numerous physiological and biochemical processes involving a variety of molecular adaptations that insects and other arthropods must undergo in acclimatizing to cold conditions, perhaps surprisingly, only two specific protein families have been exclusively connected with cold hardiness, antifreeze proteins (AFPs) and ice-nucleating proteins (INPs), both of which play key roles in all of the abovementioned cold adaptation processes. In fact, to date, AFPs and INPs are often regarded as the only two proteins uniquely associated with cold hardiness [50]. However, as indicated by the genomic studies, many proteins with important roles in other biological processes have been directly implicated in cold stress response mechanisms, including antioxidant defense system (ADS) components, heat shock proteins (HSPs), metal-binding proteins, and enzymes involved in the synthesis or degradation of cryoprotective biomolecules [18].

Since the proteomic approach is relatively new, only a limited number of proteomic studies have been conducted in the field of arthropod cryobiology. However, application of proteomics methods to study mechanisms of cryobiology

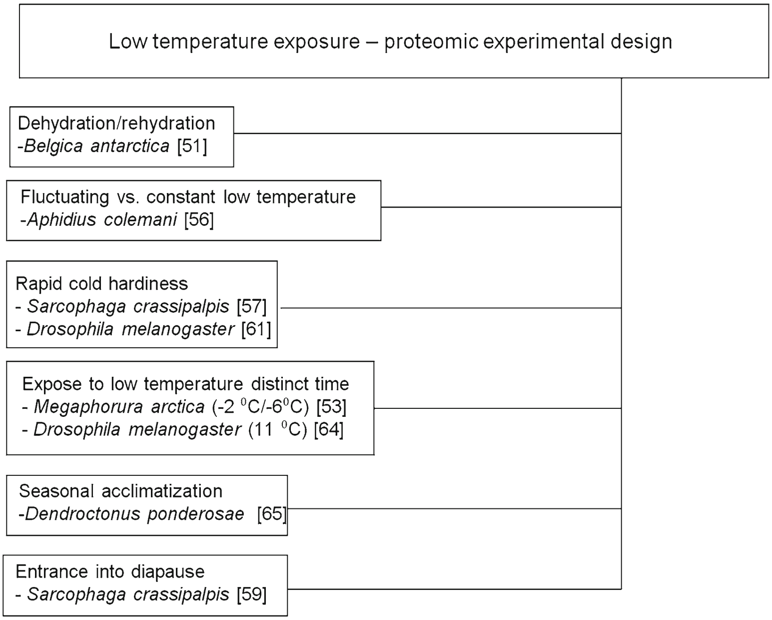


Fig. 4.2 Scheme of experimental design of proteomic studies included in this review

is increasing and is capable of providing more detailed information to complement genomic or transcriptomic approaches. In contrast with genomic approaches, proteomics has the potential to provide a broader picture of the large-scale changes in protein levels associated with cold stress and can identify proteins not previously known to be expressed during cold response. Another major advantage of proteomic methods over transcriptomics is that they enable investigation of non-model organisms if the desired target proteins possess a sufficient degree of sequence similarity with those in protein databases. Unfortunately, proteomics techniques are limited by the fact that protein databases contain comparatively less data than corresponding genomic databases, causing a large number of proteins to remain unidentified. To date, few proteomic studies have been conducted investigating the response of arthropods to low temperatures (Fig. 4.2). Moreover, direct comparison of these studies is difficult because of species divergence, variations in experimental design, and the application of different techniques, such as 2DE (two-dimensional gel electrophoresis), 2D-DIGE (two-dimensional difference gel electrophoresis), and iTRAQ (isobaric tag for relative and absolute quantitation).

In many polar arthropod species, cold hardiness is usually associated with increased resistance to other types of stress, such as desiccation [51–53]. In fact, a connection between dehydration and tolerance to subfreezing temperatures has been postulated for larvae of the Antarctic midge, *Belgica antarctica* [54], and cold

tolerance in this species was later proposed by Elnitsky et al. [55] to be dependent on a cryoprotective dehydration strategy. In 2009, Li et al. [56] performed a comparative 2DE study of *B. antarctica* larvae after cryoprotective dehydration and subsequent rehydration. As might be expected given the morphological changes involved, cytoskeletal and contractile proteins were found to be the most prevalent in both dehydrated (84 %) and rehydrated (69 %) larvae. The remaining proteins were associated with stress response mechanisms, energy metabolism, protein synthesis, and gluconeogenesis. Results of this study strongly suggest that at the proteomic level, cycles of cryoprotective dehydration/rehydration are linked to body shrinkage and cell contraction coupled with water rearrangement.

Another polar species, the Arctic springtail, *M. arctica*, also employs cryoprotective dehydration strategies in order to survive subfreezing environmental conditions [45, 57] and was recently subjected to proteomic analysis by Thorne et al. [58]. In this study, animals were acclimated at 5 °C (control) or −2 °C/–6 °C, temperatures known to induce trehalose synthesis and initiate dehydration. 2D-DIGE analysis coupled with LC–MS/MS (liquid chromatography–tandem mass spectrometry) suggested that proteins involved in protein folding (disulfide isomerase and chaperonin TCP-1), carbohydrate metabolism (glycolysis/ gluconeogenesis, trehalose production), and membrane transport (V-ATPase) are upregulated in both cold-acclimated groups. However, strikingly, proteins involved in cytoskeleton organization and four endoplasmic reticulum proteins, HSP60, Hsc70-3, calreticulin, and Grp94 (a homolog of HSP90), were found to be upregulated only in animals acclimated at −6 °C, where dehydration is more extensive. Implication of endoplasmic proteins, whose role in protein oxidation has been well documented [59], is in agreement with previous studies on this species, in which cold-induced cryoprotective desiccation was reported to be accompanied by the accumulation of hydrogen peroxide [60].

In another proteomic study based on 2DE methods, the parasitic wasp, *Aphidius colemani* [61], was exposed to fluctuating temperatures or constant cold, revealing proteins associated with energy metabolism, as well as protein synthesis and degradation (e.g., the proteasome). Of 18 identified proteins, 14 were found to be upregulated during exposure to fluctuating temperatures: eight proteins involved in energy metabolism (glycolysis, tricarboxylic acid cycle, synthesis, and conversion of ATP), two chaperones (HSP70/HSP90), one protein degradation component (proteasome), one signal transduction regulator (a regulator of phosphorylation), one actin regulatory protein, and one hypothetical protein with unknown function. Four proteins were upregulated following exposure to a constant low temperature (CLT) regime: a chitin-binding protein, a cuticular protein, the alpha subunit of ATP synthase, and a protein of unknown function. Results of this proteomic study confirmed the importance of proteins involved in energy metabolism, as well as cytoskeletal rearrangement (particularly actin). These proteins could play important roles in ensuring higher survival rates during exposure to fluctuating temperatures.

RCH has been proposed to be a general cold tolerance mechanism in insect cryobiology distinct from long-term cold acclimation which can be initiated at any developmental stage [20]. RCH-associated changes in protein profiles in the brain of pharate adults of the flesh fly, *S. crassipalpis*, were assessed by 2DE [62]. Although RCH effects are not restricted to the brain of this species, the authors focused on this tissue because it plays important roles in the initiation of glycerol synthesis [63]. Changes in expression in response to RCH treatment were observed for 14 proteins. Upregulated proteins included ATP synthase alpha subunit, a small heat shock protein (smHSP) and tropomyosin-1 isoforms 33/34, while downregulated proteins were involved in various processes, including protein degradation, transcription, cytoskeleton rearrangement, and energy metabolism. In 2011, Pavlides et al. [64] published another proteomic study on the brain of *S. crassipalpis* larvae during entrance into diapause. While cold treatment was not applied in this study, results can still be associated with cold hardiness which is an integral part of the pupal diapause syndrome in this species [65]. Total proteins and nuclear- and phosphorylation-enriched fractions from larval brains were subjected to 2DE and LC–MS/MS proteomic analysis resulting in identification of 27 proteins overall. Briefly, during entrance into diapause, 16 proteins were specifically upregulated or phosphorylated while 11 were downregulated [64]. The proteins identified in this study play known roles in cell cycle regulation, translational processing, energy storage and utilization, neural protection, cytoskeleton organization, and cell stress responses and include a transposable element of unknown function. Eleven of the proteins downregulated in total, and nuclear fractions take part in nutrient transport, cell proliferation, adult development, and aging. In addition to cold hardiness, data from this study provide insight into regulation of insect development and aging.

Proteomic methods have also been applied to investigate RCH responses in the fruit fly, *D. melanogaster*, as noted earlier, a freeze-intolerant species [66]. Only a few significantly changed protein spots were recorded using 2D-DIGE. However, four proteins of particular interest were identified as two variants of glycogen phosphorylase (GlyP), among which three spots were upregulated and one was downregulated. Subsequent Q-PCR analyses and enzymatic assays failed to reveal significant changes in the levels of GlyP mRNA or enzyme activity. However, analysis of whole animal sugar content showed a small increase in glucose concentrations (with unchanged trehalose content) suggesting complex regulation of GlyP during RCH. Results of this proteomic study are in accordance with previous transcriptomic studies on this species which failed to identify significant changes in gene expression during RCH treatment [67, 25]. Furthermore, these results are in agreement with earlier experiments on *D. melanogaster* which suggest that de novo protein synthesis occurs independently of the RCH response [68]. Another 2D-DIGE proteomic study on this species, in which adults were cold acclimated at 11 °C, showed only a mild proteomic response despite marked effects on the phenotype [69]. These results suggest that cold acclimation in the fruit fly may involve levels of protein regulation other than translation.

The cold hardiness of the coleopteran, *Dendroctonus ponderosae* (mountain pine beetle), was analyzed via a completely different proteomic approach, iTRAQ [70]. iTRAQ proteomic analyses are based on the use of four- or eight-plex isobaric tags for the relative and absolute quantification of proteins. The N-termini of proteins from different experimental groups are differentially labeled, followed by protein digestion, separation, identification, and quantification by LC–MS/MS (for further details on iTRAQ, see Zieske, 2006 [71]). Bonnet et al. [70] compared proteomic profiles of overwintering larvae sampled during cool and warm autumn months (September and November) with those sampled during cool and warm spring months (March and May). The larvae of this species gradually develop cold hardiness during the autumn months via accumulation of high levels of glycerol and initiation of freeze-avoiding mechanisms [72]. In total, over one thousand proteins were detected in autumn and spring larvae, over half of which were common to both periods. All detected proteins were classified according to their previously known functions including proteins involved in carbohydrate metabolism, cryoprotector synthesis, and cytoskeletal and cuticular processes.

Protein profiles of *D. ponderosae* larvae collected in September and November revealed 33 proteins with differential levels. In particular, exposure to low autumn temperatures appears to induce upregulation of proteins associated with energy, carbohydrate, and amino acid metabolism, as well as those involved in detoxification mechanisms. Interestingly, proteins functioning in DNA/RNA processing, signal transduction, and transport were found to be downregulated. In contrast, protein profiles from larvae collected in March and May revealed significant changes in 473 proteins. Exposure to higher spring temperatures induces upregulation of proteins associated with DNA processing, protein synthesis and modification, and cell cycle control, as well as muscle/cytoskeleton/cuticle development. In contrast, proteins involved in detoxification mechanisms, carbohydrate metabolism, and energy, lipid, and amino acid metabolism were found to be downregulated. Direct comparison of protein patterns between autumn and spring seasons revealed dramatic differences in the levels of 18 proteins, some of which displayed completely opposite expression patterns. In particular, ferritin showed a large fold change, from 4.02-fold in autumn to −17.49-fold in spring. In addition, α,α-trehalose-phosphate synthase, antennal-specific protein 3 cl (an isoprenoid biosynthesis protein), and 2-deoxyglucose-6-phosphate phosphatase displayed similar patterns as ferritin, while lipid storage droplets surface-binding protein 1 and β-tubulin followed an opposite trend. Overall, results of this study clearly implicate the influence of changes in ambient temperature in regulation of protein synthesis.

In general, all of the above-reviewed proteomic studies agree that proteins involved in energy metabolism and direct development (e.g., cell cycle) are generally downregulated, while proteins involved in carbohydrate metabolism, synthesis of cryoprotectors, chaperones (HSPs), detoxification (ADS), and metal metabolism are usually upregulated.

4.4 Metabolomics Investigations of Cold Hardiness in Insects and Other Arthropods

Metabolomics is the system-wide study of low molecular weight metabolites in order to provide a holistic view of various physiological processes and conditions [73, 74]. Although complementary to genomic and proteomic approaches, metabolomics specifically addresses changes downstream of transcription, translation, and protein activity. Thus, metabolomics has the potential to provide a unique biochemical fingerprint of a given adaptive response in an organism. Metabolomic studies are based on the detection of metabolites, usually via gas chromatography–mass spectrometry (GC–MS), liquid chromatography–mass spectrometry (LC–MS), and nuclear magnetic resonance (NMR) techniques, coupled with powerful multivariate statistical analyses methods. Although selected individual metabolomic techniques are a powerful and useful analytical tool, they have certain limitations, including a relatively low detection limit and difficulties associated with correct identification of metabolites [74]. However, in spite of the above mentioned limitations, the number of metabolomic studies of biological systems is constantly growing. Although metabolomics has not yet been extensively used to study arthropods, several important metabolomic studies on the cold hardiness and cold stress recovery in arthropods have been reported [18], predominantly relying on GC–MS and LC–MS techniques.

The low-temperature biology of arthropods (in large part insects) has been studied for more than a hundred years, and state-of-the-art metabolomic approaches confirm previously generated data, but also provide a new perspective on the molecular nature of low-temperature adaptations. Here, we review the results of several metabolomic studies on cold hardiness and cold response in arthropods. Despite the diversity of cryobiology experiments, different life stages, and cold adaptation strategies involved in the species analyzed, general metabolic patterns associated with cold hardiness and cold response can be recognized (Table 4.1). Metabolites are usually classified into four distinct groups: sugars, polyols, amino acids, and other (intermediate) metabolites. Glucose and trehalose are dominant sugars associated with responses to low-temperature exposure, followed by an increase in fructose and maltose concentrations in some species. In studies where glucose and trehalose levels did not change following cold treatment, their concentrations were already constitutively (and constantly) high [75, 76]. An increase in glycerol levels is a hallmark of cold response mechanisms, even though in many species other polyols, such as sorbitol, inositol, mannitol, and erythrol often complement multicomponent cryoprotective systems. Similar to dominant sugars (glucose and trehalose), in studies where the concentrations of glycerol, sorbitol, and inositol remain steady, the level of these polyols is already high [76, 77]. Cold stress response mechanisms generally reduce the amount of total amino acids except for alanine, which is usually increased or maintained at a constitutively high level. As an exception, metabolomics studies of cold response in *D. melanogaster* and *D. montana* did not reveal

Table 4.1 Comparative review of selected metabolomic studies on cold responses in different arthropod species

Organism (response to cold)	Treatment	Technique	Sugars			Polyols			Amino acids			Other metabolites			References
			Up	Down	Constant	Up	Down	Constant	Up	Down	Constant	Up	Down	Constant	
Class Insecta – Diptera															
Sarcophaga crassipalpis, flesh fly (freeze avoiding pupa)	RCH in adults	GC–MS	Glucose	Trehalose		Glycerol									[75]
						Sorbitol									
	Diapausing pupa freeze-avoiding	GC–MS	Glucose			Glycerol	Sorbitol		Ala	Gly		Pyruvate	Fumarate		
									Leu	Phe			Citrate		
										Pro			Isocitrate		
										Tyr					
										Asp					
Sarcophaga bullata, Gray flesh fly (freeze-avoiding pupa)	RCH+CS	GC–MS	Fructose		Ribose	Sorbitol			Val	Glu		Glucose-6P	Citrate	Succinate	[31]
	+24R in pharate adult		Glucose			Glycerol			Gly			Fructose-6P			
			Mannose			Inositol			Ser			Fumarate			
			Maltose			Erythrol			Pro			Malate			
			Trehalose			Xylitol			Leu			Glycerol-P			
						Arabitol			Ile			PO_4			
						Ribitol			Thr			Putrescine			
						Galactitol			Ala			Cadaverine			
									Phe			Gluconolactone			

(continued)

Table 4.1 (continued)

Organism (response to cold)	Treatment	Tech-nique	Sugars			Polyols			Amino acids			Other metabolites			Refer-ences
			Up	Down	Constant	Up	Down	Constant	Up	Down	Constant	Up	Down	Constant	
Belgica antarctica, Antarctic midge (freezing/ dehydration tolerant larvae)	Freezing response in final instars larvae	GC–MS			Glucose; Treha–lose	Glycerol; Erythrol; Mannitol			Ala; Asp	Gly	Ser	Urea; Succinate; Non-ionic acid			[81]
	Dessication response in final instars larvae	GC–MS				Glycerol; Erythrol	Sorbitol			Ser; Asp		GABA; Norvaline; Isocitrate; Phosphog–lycerate; Succinate; Nonionic acid			
Drosophila melanogaster, fruit fly (freezing intolerant)	CS	NMR	Maltose; Trehalose					Glycerol; Inositol; Sorbitol				Phospho-choline	Fatty acid lipid-like metabo lite		[77]
	RCH+CS	NMR	Maltose; Trehalose; Glucose					Glycerol; Inositol; Sorbitol	Ala						
D. melanogaster, fruit fly (freezing intolerant)	CS inbreeding versus outbreeding	NMR	Maltose; Trehalose; Glucose			Glycerol; Inositol			vPro	b-Ala			NAD+	3-hydroxy kynurenine; Galactoside	[78]

D. montana (freeze avoiding)	Diapausing adult during winter period	GC–MS / LC–MS	Glucose Trehalose			Myo-inositol			Pro	Cys Gly His Ile Leu Lys Met Ser			Lactate		[79]
Heleomyza borealis (freeze-tolerant larvae)	CS+48R	NMR	Glucose								Arg Glu/Gln Pro Asp Ala Val Tyr			Citrate Formate Lactate Pyruvate NAD+ ATP	[82]
Class Insecta - Lepidoptera															
Cydia pomonella, codling moth (freeze-avoiding)	Fifth instar diapausing larvae in winter	GC–MS / LC–MS	Fructose Glucose	Glycogen	Trehalose (high)	Sorbitol Glycerol Mannitol Arabinitol			Ala	Glu	Pro				[76]
Ostrinia nubilalis, European corn borer (freeze tolerant)	Fifth instar diapausing larvae exposed to subzero tempera-tures	NMR				Glycerol			Ala	Pro Ser Val Ile Leu Gln		Acetate	Citrate Lactate Putrescine		Unpub-lished data

(continued)

Table 4.1 (continued)

Organism (response to cold)	Treatment	Technique	Sugars			Polyols			Amino acids			Other metabolites			References
			Up	Down	Constant	Up	Down	Constant	Up	Down	Constant	Up	Down	Constant	
Class Insecta - Hymenoptera															
Venturia canescens, parasitoid wasp (freeze-intolerant)	Adult developing at 17 °C	GC–MS	Glucose						Ala			Glucose-6P			[83]
			Mannose						Gly			Putrescine			
			Fructose						Leu			Cadaverine			
									Ile			Glycerate			
Class Arachnida – Trombidiformes															
Tetranychus urticae, two-spotted spider mite (cold hardy)	Diapausing females acclimated to 5 °C	GC–MS	Glucose	Fructose	Galactose	Mannitol	Add-onitol	Glycerol		Gln	Ala	Glucono-lactone	Citrate		[84]
			Maltose	Ribose		Inositol				Gly	Glu		Malate Fumarate		
				Mannose		Sorbitol				Iso			Glycerol-3P		
										Leu					
										Orn					
										Phe					
										Pro					
										Ser					
										Thr					
										Trp					
										Val					

Only the most representative data are extracted; for more information on each study, please refer to a given reference
RCH rapid cold hardening, *CS* cold shock, *24R* 24 h recovery, *48R* 48 h recovery, *GC-MS* gas chromatography–mass spectrometry, *LC–MS* liquid chromatography–mass spectrometry, *NMR* nuclear magnetic resonance

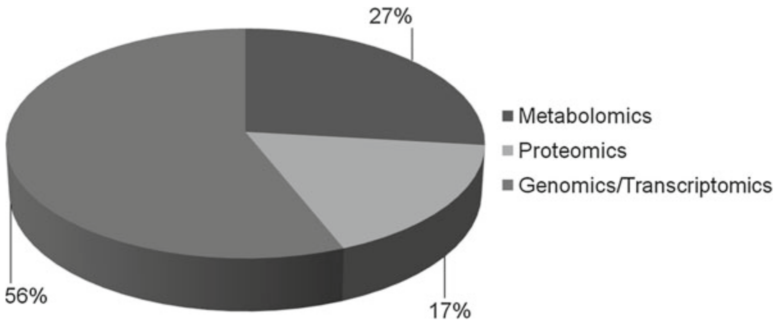


Fig. 4.3 Relative proportion of different “omics” in insect cold hardiness research. Graphical representation of all published works cited in this chapter that utilized “omics” techniques to investigate cold adaptation responses in insects and arthropods. The relative proportion of studies using metabolomics, proteomics, or genomics/transcriptomics is shown

an increase in alanine concentrations, but rather proline [78, 79]. As for other commonly detected metabolites possibly involved in the response to cold stress, intermediates of glycolysis and the Krebs cycle usually decrease, while selected intermediates of the pentose phosphate pathway, urea, and polyamines and synthesis of cryoprotectants increase in selected species (Table 4.1), which is in agreement with previous studies [80].

4.5 Concluding Remarks

Insect cold hardiness remains an unsolved, understudied phenomenon. Many different strategies are utilized by a diverse array of species attempting to thrive in harsh, cold environmental conditions. While we are still far from answers to many questions surrounding insect cold hardiness, the recent development of “omics” technologies is rapidly enabling researchers to improve our knowledge significantly. Although our understanding is improving (Fig. 4.3), we have a long way to go toward development of plausible models of these processes in their entirety. In fact, the beautiful complexity of cold hardiness phenomena is a wonderful example of the interplay of multiple biomolecules in their roles as part of a larger system or network, waiting to be solved in the light of systems biology.

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Chapter 5
Selenocysteine Extinctions in Insects

Marco Mariotti

Abstract Selenocysteine (Sec) is a nonstandard amino acid present in a minor fraction of proteins, called selenoproteins. Sec is an analog of cysteine (Cys) with selenium replacing sulfur. Sec is inserted during translation, in correspondence to specific UGA codons (normally stop signals) recoded in the presence of peculiar RNA structures. Sec is generally used in key sites of oxidoreductase enzymes. Its biosynthesis and insertion require a set of trans-factors collectively called Sec machinery, including a Sec-tRNA and a Sec-specific elongation factor.

Sec is found all across the tree of life, with a scattered distribution. Species that do not encode selenoproteins often replace their function with Cys homologues. In prokaryotes, Sec is present in a minor fraction of extant organisms, presumably due to frequent losses from a Sec-containing ancestor. Sec is much more common among eukaryotes and particularly metazoans.

Insects constitute a convenient lineage to study the dynamic evolution of the Sec trait. While other animals have more than 12 different families of Sec enzymes, many insects were recently discovered to be completely devoid of them. After the split with the rest of the arthropods, a progressive selenoproteome reduction occurred in insects through gene losses and Sec-to-Cys conversions. In many lineages, this culminated in complete loss of Sec coding ability (Sec extinction), evident from the lack of complete Sec machinery in model insect genomes. At least five phylogenetically independent events of Sec extinctions happened in insects. The majority occurred in Endopterygota. Here, the entire orders of Coleoptera, Lepidoptera, and Hymenoptera appear devoid of selenoproteins. More recent events affected the species, *Drosophila willistoni* (Diptera) and *Acyrthosiphon pisum* (Hemiptera). To justify the independent Sec extinctions in insects, it is believed that

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this lineage went through a relaxation of the selective constraints to maintain Sec. Its exact nature remains rather speculative, but it is probably related to peculiarities in the antioxidant systems of insects compared to other animals.

Keywords Selenocysteine • Selenoprotein • Recoding • Evolution • Antioxidant defense

Abbreviations

Cys	Cysteine
EFSec	Selenocysteine elongation factor
ER	Endoplasmic reticulum
ERAD	Endoplasmic reticulum-associated degradation
GPx	Glutathione peroxidase
Msr	Methionine sulfoxide reductase
MsrA	Methionine-S-sulfoxide reductase
MsrB	Methionine-R-sulfoxide reductase (selenoprotein R)
PSTK	Phosphoseryl-tRNA[ser]sec kinase
Pyl	Pyrrolysine
ROS	Reactive oxygen species
SBP2	SECIS binding protein 2
Sec	Selenocysteine
SECIS	Selenocysteine insertion sequence
SECp43	tRNA selenocysteine 1-associated protein 1
SecS	Selenocysteine synthase
SerRS	Seryl-tRNA synthetase
SPS	Selenophosphate synthetase
SPS1	Selenophosphate synthetase 1
SPS2	Selenophosphate synthetase 2
TR	Thioredoxin reductase
tRNAsec	Selenocysteine transfer RNA

5.1 Introduction

5.1.1 Nonstandard Amino Acids

Proteins are macromolecules central to the biological functions of all living organisms for their enzymatic, signaling, and structural roles. Proteins are linear polymers composed of amino acids, which are building blocks that differ only in their side chains. Despite some differences in the actors involved, the process of natural protein synthesis (translation) follows the same general principles in all organisms. The coding sequences within mRNAs are read in triplets (codons), determining at

UUU	Phe	UCU	Ser	UAU	Tyr	UGU	Cys
UUC	Phe	UCC	Ser	UAC	Tyr	UGC	Cys
UUA	Leu	UCA	Ser	UAA	Stop	UGA	Stop/Sec
UUG	Leu	UCG	Ser	UAG	Stop	UGG	Trp
CUU	Leu	CCU	Pro	CAU	His	CGU	Arg
CUC	Leu	CCC	Pro	CAC	His	CGC	Arg
CUA	Leu	CCA	Pro	CAA	Gln	CGA	Arg
CUG	Leu	CCG	Pro	CAG	Gln	CGG	Arg
AUU	Ile	ACU	Thr	AAU	Asn	AGU	Ser
AUC	Ile	ACC	Thr	AAC	Asn	AGC	Ser
AUA	Ile	ACA	Thr	AAA	Lys	AGA	Arg
AUG	Met	ACG	Thr	AAG	Lys	AGG	Arg
GUU	Val	GCU	Ala	GAU	Asp	GGU	Gly
GUC	Val	GCC	Ala	GAC	Asp	GGC	Gly
GUA	Val	GCA	Ala	GAA	Glu	GGA	Gly
GUG	Val	GCG	Ala	GAG	Glu	GGG	Gly

Fig. 5.1 The universal genetic code, used by all metazoans for nuclear genes. Stop codons are highlighted in orange. UGA is normally a stop, but it is recoded to Sec in the genes encoding selenoproteins. Almost all known metazoans have selenoproteins, with exceptions found only in certain insects and nematodes

each position the nature of the amino acid residue inserted in the growing peptide. The set of rules underlying protein translation can be expressed as a table with univocal correspondences between codons and amino acids: the genetic code. A few codons specify termination of the translation process and subsequent release of the complete peptide (stop signals). The genetic code is remarkably conserved: the great majority of organisms use the same, "universal" genetic code (Fig. 5.1). All alternative genetic codes (including the mitochondrial codes) are still very similar (see [1]). In all known living organisms, the same 20 *standard* amino acids are inserted. This repertoire is expanded by posttranslational modifications, such as phosphorylation, acetylation, glycosylation, and many more.

Two additional amino acids exist, and they are generally overlooked by most biologists: selenocysteine (Sec, the 21st amino acid) and pyrrolysine (Pyl, the 22nd amino acid). Like standard amino acids, Sec and Pyl are inserted co-translationally while bound to a tRNA of their own. Yet, they do not have a fully dedicated codon in any known genetic code. Instead, they are inserted through *recoding* events: translation makes punctual exceptions for a single codon (or rarely for a few).

These two nonstandard amino acids are not found in all organisms. Pyl seems very rare and has been detected only in some methanogenic archaea and a few bacterial species [2]. It is inserted in correspondence to UAG codons (normally stop signals) in the active site of methylamine methyltransferases. However, since no precise signals are required for Pyl recoding of UAG codons [3], nonspecific insertion of Pyl is likely to occur in many other proteins in these organisms. Because of its absence from eukaryotic genomes, pyrrolysine is not further treated here; for additional reading, we recommend [2, 4, 5].

Sec is found scattered across the three domains of life: bacteria, archaea, and eukaryotes. It is inserted in response to specific UGA codons corresponding to key sites of enzymes. In these transcripts, peculiar RNA structures inform the ribosome of recoding UGA to Sec. The mechanisms of Sec recoding and biosynthesis exhibit both common aspects and substantial differences among the three domains, briefly presented in the next section. Proteins containing at least one Sec residue are called *selenoproteins*. Sometimes, this term is also used to indicate proteins with unspecific selenium-containing amino acids, resulting from selenium spuriously entering sulfur pathways. These "unspecific selenoproteins" are not considered here.

5.1.2 Recoding: Expansions of the Genetic Code

Like Pyl, Sec is inserted in response to recoded stop codons (with a single documented exception in the ciliate *Euplotes*, where UGA normally codes for cysteine (Cys) [6]). The phenomenon of recoding, defined as the programmed deviation from the standard translation process, is not limited to Sec and Pyl; many diverse classes are known (see [4] for a detailed description). Sec and Pyl are considered a form of stop codon readthrough (or stop redefinition). Other cases of stop codon readthrough are known, in which standard amino acids are inserted via recognition by a charged tRNA. Ribosomal frameshifting is another recoding phenomenon in which the translating ribosome breaks the standard 3-by-3 readout, changing the current open reading frame. By contrast, transcriptional slippage is an event that happens during the synthesis of RNA, involving the dissociation and reassociation of the nascent mRNA from the DNA template. Since it results in transcripts with inserts of one or more nucleotides at a specific site, its final effects are analogous to ribosomal frameshifting. Other cases of recoding involve the bypassing of a stretch of RNA during translation (also called ribosomal hopping or skipping) or the synthesis of two separated peptides within the same open reading frame, despite the absence of a stop codon (StopGo). Most characterized recoding events depend on sequence elements acting at the level of RNA [4]. Examples are "slippery" sequences that promote defective template reassociation or secondary structures like stems and pseudoknots, which often work by stalling translating ribosomes. Instances of recoding are found throughout all living organisms and beyond. In viruses, they are very common, presumably because they allow enriched diversity of gene products while keeping a compact genome. There is increasing evidence that animal genomes are also rich in recoded genes, as testified by the current release of the Recode-2 database [7].

In insects in particular, recent analyses have revealed an abundance of (non-Sec) stop codon readthrough. In Lin et al. [8] and Jungreis et al. [9a], the *Drosophila* genomes were scanned using a computational method that searched for strong evolutionary signatures indicating translation. After a rigorous analysis to exclude alternative explanations, this resulted in a list of 283 readthrough genes [9a]. A more recent study used ribosome profiling (deep sequencing of ribosome-protected fragments) to examine translational readthrough in *Drosophila melanogaster* [9a].

This analysis confirmed readthrough of a limited portion of previous candidates and additionally found 307 genes with ribosome profiling support but no clear evolutionary signature. Lately, the method used in [9a] was applied to recently sequenced *Anopheles* genomes, also resulting in hundreds of strong readthrough candidates [10]. Using more indirect methods, it was estimated that readthrough is much more abundant in all insects (and also in the crustacean *Daphnia pulex*) compared to vertebrates and most other animals [9a]. The increased importance of stop codon readthrough for insects, however, does not pertain to selenoproteins, which instead follow an opposite trend—as described later in this chapter.

Recoding events represent expansions of the genetic code. Often, we can find a rationale in protein regulation, both for feedback systems and stoichiometry. For example, the synthesis of full-length (functional) ornithine decarboxylase antizyme (inhibitors of polyamine biosynthesis) requires a frameshifting event which is modulated by the concentration of polyamines [11]. In viruses, recoding is often used to maintain the right proportion of structural proteins and replication enzymes (e.g., gag-pol in HIV) [4]. Stop codon readthrough is used in fungi and other eukaryotes to add peroxisome localization signals to a precise fraction of synthesized enzymes [12]. The magnitude of translational readthrough in insects also suggests the importance of this process to diversify gene products and allow the dynamic expansion of proteomes.

The case of recoded nonstandard amino acids, and especially Sec, seems to differ in purpose. The *nature* of the special residue inserted at these sites appears to be the main reason for these recoding events. Sec is utilized only in very specific sites of proteins, reflecting a deep level of specialization compared to standard amino acids. Sec insertion always happens only for a narrow and specific fraction of the total genes in any species. With very few exceptions, there is only one Sec site in each selenoprotein. The reasons for such specialization are tightly linked to the biochemical properties of Sec.

5.1.3 Selenocysteine Versus Cysteine

Sec is analogous to Cys but contains selenium in place of sulfur, thus exposing a selenol instead of a thiol group (Fig. 5.2). Cys and Sec have roughly similar properties, but also important differences. For the great majority of selenoproteins, Cys

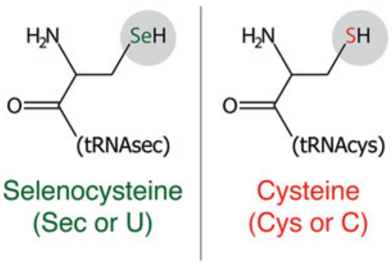


Fig. 5.2 Structures of selenocysteine and cysteine

homologues are known [13]. These are standard proteins in which Cys aligns to the Sec site of a known selenoprotein, encoded by an orthologous or paralogous gene. While Cys is used for many functions in the cell, Sec is almost invariantly found in the active site of oxidoreductases [14]. In these enzymes, usually Sec substitutes for one Cys involved in thiol/disulfide exchange reactions, which thus become selenol-thiol/selenosulfide reactions.

Sec insertion appears to be inherently inefficient compared to standard amino acids [15]. Moreover, the synthesis of selenoproteins requires the conservation and concerted regulation of several Sec-specific factors, adding up to the "costs" of Sec. To justify its use, Sec must then provide some selective advantage over Cys in selenoproteins. This is consistent with the results from studies exploring artificial Sec/Cys mutations in the thioredoxin reductase selenoprotein family [16, 17]; here, Sec generally shows higher catalytic efficiency than Cys. The exact nature of the advantage provided by Sec is highly debated in the selenium community (see [17–20]) and may well differ for different selenoprotein families. Explanations have invoked the greater nucleophilicity of selenolate versus thiolate, or greater electrophilicity of the selenium atom in selenosulfide upon nucleophilic attack, or increased leaving group ability, or better resistance to inactivation by irreversible oxidation. In more general terms, it is commonly accepted that Sec is favored over Cys for its higher reactivity and catalytic efficiency. However, the advantage of Sec over Cys seems limited to a narrow set of functions. Selenoproteins constitute at best a minute fraction of the total proteome of any organism. Sec is unfavorable in most protein sites, probably again for its high reactivity. Sec insertion through UGA recoding may be seen as natural selection's compromise to have Sec in key redox enzymes, while avoiding its insertion across the whole proteome.

Not all species utilize selenoproteins. Although the Sec utilization trait is proposed to be ancestral, it has been lost independently in many lineages. Insects offer the possibility to study the dynamics of this genomic process. In fact, while selenoproteins are generally present and essential in animals, several insect lineages are devoid. This marks a clear distinction from vertebrates, which tightly conserve their selenoprotein genes, and highlights important differences between insects and other animals.

5.2 Sec Machinery

The term *Sec trait* is often used to designate the capacity to code for Sec, conferred by a specific set of genes. We collectively call these genes *Sec machinery*, including those necessary for the synthesis of Sec and its directed insertion during translation of selenoprotein transcripts. In this section, we provide an essential summary of these processes; for further information, we recommend reading [4, 21, 22].

5.2.1 Eukaryotic Sec Biosynthesis

The biosynthesis of Sec takes place on its own tRNA (Fig. 5.3a). tRNAsec exhibits many peculiar characteristics compared to standard tRNAs [23, 24], the most prominent of which is a long extra arm. tRNAsec is recognized by the standard seryl-tRNA synthetase (SerRS), and so it is initially charged with serine [25]. This is then converted to Sec in two steps. First, serine is activated through phosphorylation by phosphoseryl-tRNA[ser]sec kinase (PSTK) [26] to phosphoserine. Secondly, selenocysteine synthase (SecS or SepSecS, previously known as SLA/LP) catalyzes the conversion of the phosphoserine on tRNAsec to Sec, using selenophosphate as the selenium donor [27].

Selenophosphate is provided by selenophosphate synthetase (SPS, called SelD in prokaryotes), which produces it from selenide and ATP [28]. In metazoans, including *D. melanogaster*, two SPS genes are found. SPS2 is the enzyme that actually performs selenophosphate synthesis, and intriguingly it is itself a selenoprotein, containing Sec on its N-terminal domain. The protein SPS1, although similar in

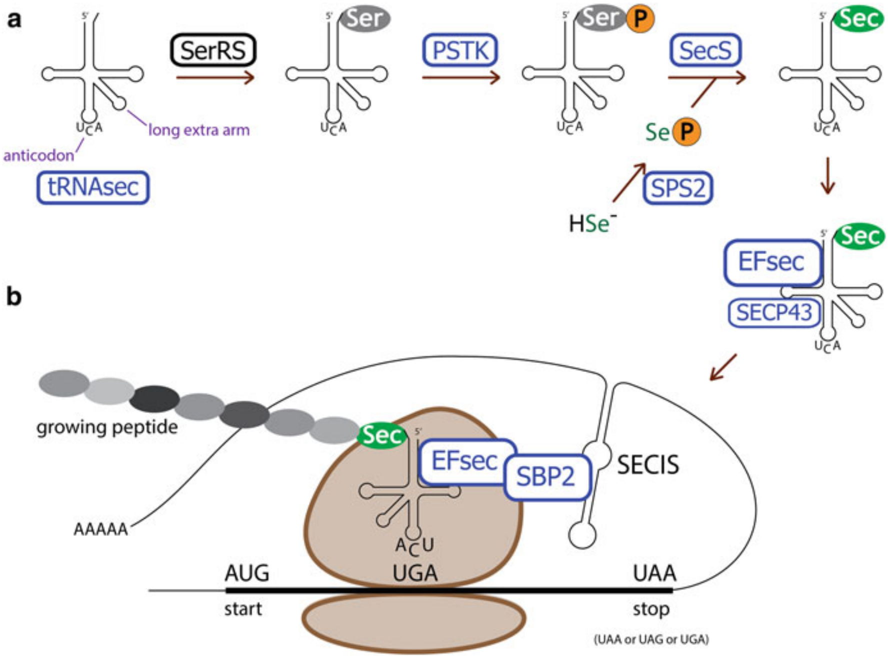


Fig. 5.3 Mechanisms of eukaryotic Sec synthesis and insertion. (**a**) Sec synthesis on its own tRNA. (**b**) Sec insertion through recoding during selenoprotein translation. Sec machinery factors are displayed in blue. *Sec*, selenocysteine; *tRNAsec*, Sec tRNA; *SerRS*, seryl-tRNA synthase; *PSTK*, phosphoseryl-tRNA[ser]sec kinase; *EFsec*, selenocysteine elongation factor; *SECP43*, tRNA selenocysteine 1-associated protein 1; *SBP2*, SECIS binding protein 2; *SECIS*, selenocysteine insertion sequence

sequence to SPS2 (but with no Sec), was shown not to catalyze this reaction [27, 29], but its function is still unknown today. It is believed to be unrelated to Sec [30a, 30b] and possibly linked to vitamin B6 biosynthesis [31]. For these reasons, here SPS1 is not considered as part of Sec machinery.

5.2.2 *Eukaryotic Sec Insertion*

The necessary signal for UGA to Sec recoding is a structure called the selenocysteine insertion sequence or SECIS element. In eukaryotes, this is a stem-loop structure found in the 3'UTR of selenoprotein transcripts [32, 33] (Fig. 5.3b) which consists of two helices separated by a loop, with a conserved core found at the base of helix 2 [34, 35] (Fig. 5.4). The core (or quartet) consists of two non-Watson-Crick AG

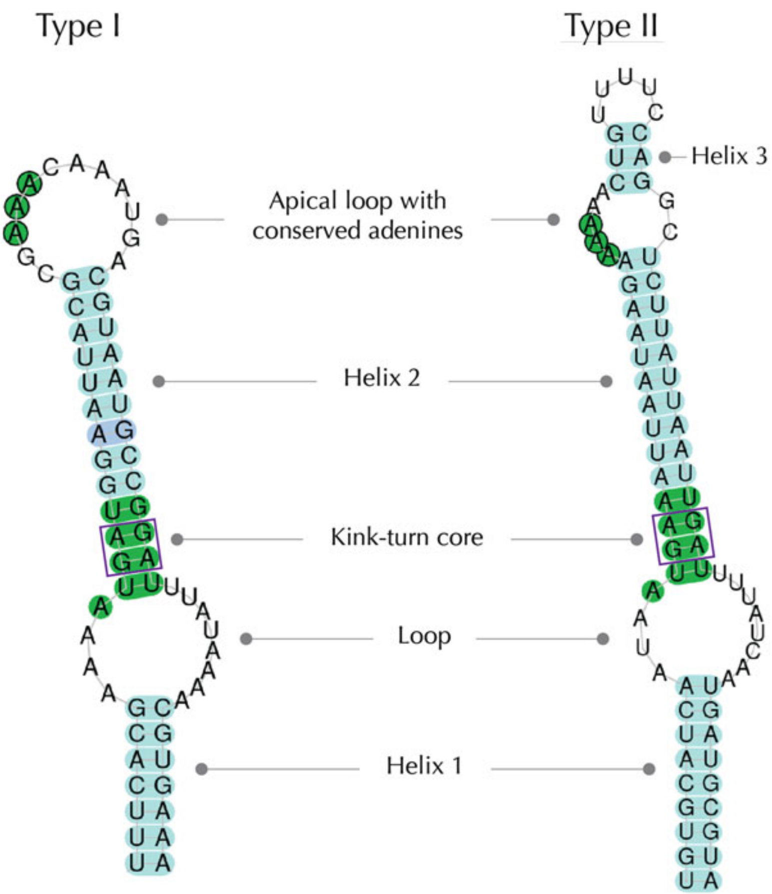


Fig. 5.4 Types of eukaryotic SECIS elements. Conserved nucleotides are highlighted in green. (**a**) Type I SECIS from the *Strigamia maritima* SelP gene. (**b**) Type II SECIS (more common) from the *S. maritima* MsrB gene. Drawn with SECISearch3 [36]

pairs forming a peculiar RNA motif known as kink-turn [37]. At the top of helix2, there is an apical loop with additional conserved nucleotides. SECIS elements are classified into two types [38], with type II (most widespread [39]) possessing an additional helix in the apical loop. For a review of conserved features and binding determinants of SECIS elements, see [37, 39–41].

SECIS elements in selenoprotein transcripts are recognized by SECIS binding protein 2 (SBP2), a major player in Sec decoding [42–44]. The two SECIS forms appear to be functionally equivalent in vertebrates, but not in insects; *D. melanogaster* selenoproteins present only type II SECIS elements, and its SBP2 protein was shown to have poor affinity toward type I forms [45].

The other key player in Sec decoding is the selenocysteine elongation factor, EFsec [46]. EFsec contains a large N-terminal region with high homology to EF-tu, a standard elongation factor. EF-tu is involved in the delivery of charged tRNAs to the A site of the ribosome, allowing peptide bond formation and thus elongation. EFsec performs an analogous function, but it is used uniquely for Sec UGA positions, binding specifically tRNAsec charged with Sec [47]. SBP2 seems to act as a bridge between all the players; besides SECIS elements, it contacts Sec-tRNAsec bound to EFsec [48, 49] and also the ribosome [50] (Fig. 5.3b). This complex is likely to be assembled in the nucleus [21].

The protein SECp43 was also identified as part of the Sec machinery, since it binds tRNAsec [51]. SECp43 is believed to form a multi-protein complex with EFsec (Fig. 5.3a), possibly regulating its nuclear shuttling [52]. SECp43 is also involved in methylation at position 34 of tRNAsec [52], which seems to play a role in the preferred expression of a subset of stress-related selenoproteins in vertebrates [53].

5.2.3 Differences in Prokaryotic Sec Machinery

The process of Sec biosynthesis is essentially identical in eukaryotes and archaea, with archaeal proteins homologous to eukaryotic SPS, PSTK, and SecS [54]. In contrast, bacteria have no homologue to PSTK; the serine charged on tRNAsec is transformed to selenocysteine without prior activation by phosphorylation. The bacterial selenocysteine synthase (called SelA) has very different sequence and structure than its eukaryotic/archaeal counterpart [55], casting doubt on their common descent.

The process of Sec insertion (translational recoding) presents stronger differences across the three domains of life. In archaea, SECIS elements are found in the 3’UTR, as in eukaryotes (although there is one documented case where, surprisingly, it is located in the 5’UTR [56]). However, their sequence and structure have very poor similarity with eukaryotic SECIS elements, primarily for the absence of a kink-turn motif and conserved quartet. To date, despite all efforts, no SECIS binding protein has been identified in archaea, leaving open the question of how the SECIS and the Sec site communicate [57].

Bacterial SECIS elements reside within the coding sequence, next to (or including) the Sec-UGA [58]. Their structure does not resemble either archaeal or eukaryotic

SECIS elements [40]. Bacterial EFsec (called SelB) recognizes SECIS elements directly through its C-terminal domain, whereas its N-terminal domain works, again, as an elongation factor. Despite these differences, the main core of the Sec machinery is similar enough in the three domains to suggest a common descent (tRNAsec, SelB/EFsec, SelD/SPS). For this reason, it is generally believed that Sec evolved just once during evolution.

5.3 Selenoproteins Across the Tree of Life

Only ~15–30 % of sequenced bacterial genomes possess the Sec trait, with a rather scattered pattern [59, 30b]. In archaea, Sec was detected only in the Methanococcales and Methanopyri lineages [57]. Mostly, the Sec trait distribution in prokaryotes seems to be the result of frequent losses from a common Sec-containing ancestor. Analyzing the phylogenetic structure of species and the sequence signal on Sec machinery genes, it is evident that horizontal transfer of the Sec trait also played a role in bacteria [59, 30b]. The prokaryotic Sec machinery is encoded by genes often forming a cluster, making this event relatively easy.

This is not the case in eukaryotes, for which there is no documented case of Sec trait loss and reacquisition. When very distant lineages are considered (typically in protists), the Sec trait distribution still appears scattered [30b], presumably due to multiple losses of the Sec trait.

Green algae have been found to be abundant in selenoproteins [60–62]. In contrast, all sequenced land plant genomes (Embryophyta) are devoid of Sec. All analyzed fungi also lack Sec [63]. By contrast, selenoproteins have been found in all analyzed Kinetoplastida (parasites including *Leishmania* and *Trypanosoma*) [64, 65], as well as in diatoms, Mycetozoa (amoebas) [62], and ciliates [6]. Selenocysteine has been found only in a subset of the remaining sequenced protozoans. For example, selenoproteins were detected in *Plasmodium* [66], *Naegleria* [67], *Emiliania* [68], and *Aureococcus* [69], but not in *Entamoeba*, *Trichomonas*, or *Giardia* [30b].

Animals generally possess the Sec trait and have many selenoprotein genes (>15). The only known metazoans that have few or none are found within certain protostome lineages, for example, *D. melanogaster* (fruit fly) has three, *Apis mellifera* (honeybee) has none, and *Caenorhabditis elegans* (nematode worm) only one. Before describing these lineages, we will delineate selenoproteins in other animals, for they reflect more accurately the metazoan ancestral state.

5.4 Selenoproteins in Metazoans

Most metazoan selenoproteins have been characterized in vertebrates, in which they cover many essential roles. The full selenoproteomes of mouse and human consist of 24 and 25 selenoproteins, respectively [70]. Their only difference is GPx6, a selenoprotein in human that was converted to a Cys homologue in mouse and rat.

A few other Sec-to-Cys conversions have been reported in vertebrates. Together with some selenoprotein gene losses and duplications, these events shaped the vertebrate selenoproteome from an ancestral state of 28 Sec-containing genes [71]. Despite few changes, the number of selenoprotein genes found in all analyzed tetrapods is very similar, 24–25. Among all vertebrates, only bony fishes seem to have a significantly higher number of selenoprotein genes (35–38) [71], likely through the frequent whole genome duplications in this lineage. Selenoproteins are well conserved in vertebrates, with evidence of strong purifying selection acting on Sec UGA sites to prevent conversion to Cys [72].

The great majority of the vertebrate selenoprotein families are shared with primitive animals, such as Cnidaria, Placozoa, and even Porifera [73] (Fig. 5.5). Therefore, the last common ancestor of metazoans is expected to have possessed a rich selenoproteome, with ~18 different selenoprotein families. The analysis of selenoproteins in genomes of certain protostomes (such as mollusks and annelids [73]) shows that the same can be said for their last common ancestor and therefore also for the first bilaterian animal—the last common ancestor of vertebrates and insects. It is evident that the poor number of selenoproteins in insects and nematodes is a derived, rather than an ancestral feature.

The analysis of the centipede genome has recently shed light on the selenoproteome of ancestral arthropods. Twenty selenoprotein genes belonging to 14 different families have been found in *Strigamia maritima* [79] (Fig. 5.5). Other sequenced noninsect arthropods (*D. pulex*, *Ixodes scapularis)* possess a similar selenoproteome, differing only in a few Sec families (unpublished results).

Based on this, the ancestral arthropod is predicted to possess a set of selenoproteins quite similar to vertebrates. This implies that the low number of selenoprotein genes in insects has to be ascribed to events that occurred after the split with the rest of the arthropods [74]. Also, it indicates that the depletions in insects and nematodes are phylogenetically independent phenomena, which occurred long after their split.

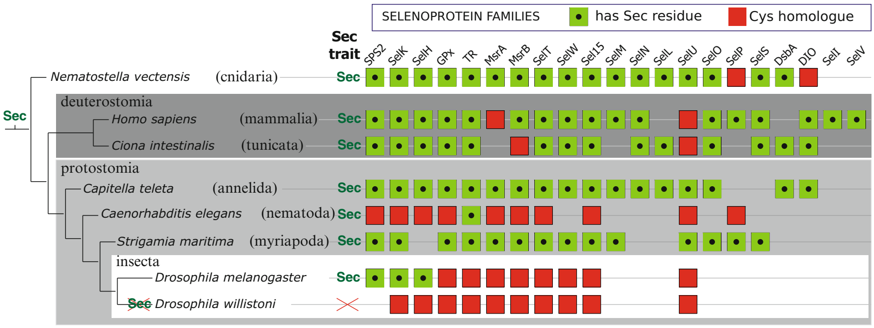


Fig. 5.5 Selenoprotein families across metazoan lineages. Insects are expanded in Fig. 5.7. All metazoan selenoprotein families are shown here, except: SelJ (found with Sec only in fish, echinoderms [77], and amphioxus [73]), Fep15 (in fish [78]), and AphC (in Porifera [73]). When both Sec and Cys forms of a selenoprotein family are present in a species, Sec is displayed. The figure summarizes data from [73] and unpublished results obtained with the program Selenoprofiles [76]

5.4.1 Functions of Metazoan Selenoproteins

The process of oxidative defense (or antioxidant defense) is responsible for maintaining redox homeostasis by clearing out oxidizing agents such as reactive oxygen species (ROS). Although bursts of oxidants are sometimes used for signaling and expression regulation [80], they generally represent a form of damage, potentially compromising the function of proteins and the integrity of DNA. Most metazoan selenoproteins are oxidoreductases, and many are related to oxidative defense. They work as reductant enzymes, either acting directly on the oxidants, or reducing other antioxidant molecules, or repairing the damage on oxidized proteins.

Thioredoxin reductases (TR) are enzymes that catalyze the reduction of thioredoxins at the expense of NADPH. Thioredoxins are small oxidoreductase proteins, important in the redox equilibrium of all living cells [80]. They act directly as reductants or as electron donors to other antioxidant enzymes such as peroxiredoxins. The TR proteins are the only known enzymes responsible for the reduction of thioredoxins [81]. Tetrapods contain three TR genes, all of which contain selenocysteine as a penultimate residue [71, 80]. The TR enzymes are spread across all living organisms, but Sec-containing TR are limited to eukaryotes.

Glutathione peroxidases (GPx) are enzymes catalyzing the reduction of peroxides at the expense of glutathione, an important antioxidant tripeptide. In vertebrates, glutathione is then reduced by a dedicated enzyme, glutathione reductase. The GPx enzymes are found spread across the whole tree of life, with Sec-containing forms found both in bacteria [82] and eukaryotes [83]. The TR and GPx enzymes are important components of thioredoxin and glutathione systems, very ancient tools that are central to redox homeostasis.

Methionine sulfoxide reductases (Msr) are ubiquitous enzymes that reduce methionine sulfoxides back to methionine, thus repairing an effect of oxidative damage in proteins. Two families of Msr exist in multicellular eukaryotes, MsrA and MsrB (also called SelR), specific for the S and R enantiomers of methionine sulfoxide, respectively [84]. Both MsrA and MsrB are found as selenoproteins in many metazoans (Fig. 5.5). Vertebrates possess Sec-containing forms of MsrB (as well as Cys paralogues), but only Cys homologues for MsrA.

The metazoan genes *SelH* (present also in fruit fly, detailed later), *SelT*, *SelW*, and *SelV* encode selenoproteins that can be grouped into a single superfamily. They all present a thioredoxin-like fold and an active site with a redox-box motif, Cys-X-X-Cys, where one Cys is replaced by Sec. All these proteins are then expected to act as oxidoreductases through a selenide-sulfide/selenol-thiol switch and are likely to be linked to antioxidant defense [85].

Some metazoan selenoproteins are found in oxidative protein folding pathways. DsbA is a family of protein disulfide isomerases that introduces disulfide bonds into nascent proteins in *Escherichia coli*. Sec-containing DsbA-like proteins are found in several metazoans [86] (Fig. 5.5). The selenoproteins Sel15, Fep15, and SelM (all phylogenetically related) reside in the endoplasmic reticulum (ER) and are proposed to have a role in the control of the correct folding of proteins [87]. Selenoproteins

SelK (also present in fruit fly, detailed later) and SelS are likely to work in the pathway of ER-associated degradation (ERAD), which targets misfolded proteins and signals them to the proteasome for disposal [88, 89]. The remaining metazoan selenoproteins carry out very diverse functions, for example, Se transport for SelP, maturation of thyroid hormones for DIO1-2-3, and selenophosphate synthesis for SPS2. Some are still completely uncharacterized.

As described below, insects possess a very limited subset of all metazoan selenoproteins, and many possess none at all. This is due to the progressive selenoproteome reduction that occurred during their evolution, often culminating in complete loss of the Sec trait. Throughout this process, the selenoprotein functions mentioned above were either lost or decoupled from selenium usage through Sec to Cys conversions.

5.5 Selenoproteins in Insects

5.5.1 *Selenoproteins and Sec Machinery in* Drosophila melanogaster

The genome sequence of the model fruit fly (*D. melanogaster*) was published in 2000 [90]. Its full set of selenoproteins was first described 1 year later [91, 92]. This organism has only three selenoproteins, SelH, SelK, and SPS2. We describe each of them in the following paragraphs.

SelH (also called BthD, flybase id FBgn0030501) contains a thioredoxin-like fold, typical of many selenoproteins. Sec is found in a redox-box motif, Cys-X-X-Sec. SelH appears to be involved in antioxidant response via the glutathione system. The RNAi-induced knockdown in *Drosophila* embryos and S2 cell lines reduced viability and decreased antioxidant capacity, while its overexpression rescued the effects of glutathione depletion [93]. Its mammalian Sec-containing homologue was shown to possess glutathione peroxidase activity in vitro [94]. In another study, murine HT22 cells were transfected to overexpress human SelH [95]. These cells exhibited enhanced resistance to depletion of glutathione by oxidative treatment with buthionine sulfoximine. Increased expression of a number of genes was detected, involved both in glutathione biosynthesis and its conjugation for detoxification. Considering that SelH localizes in the nucleus [94, 95] and contains an AT-hook DNA-binding domain [95], it was proposed that its role (at least in mammals) is to sense the redox state and transcriptionally regulate glutathione pathways. SelH possesses two non-Sec paralogues in *D. melanogaster* and also in the majority of other sequenced *Drosophila* genomes. SelH2 bears an arginine aligned to the Sec site, while SelH3 bears a Cys. They both seem to be derived from duplications of SelH at different times; we mapped the origin of SelH3 to the root of Diptera and SelH2 to the root of *Drosophila* (unpublished results). However, neither SelH2 nor SelH3 conserve intact the redox-box motif of SelH and thus may have distinct roles.

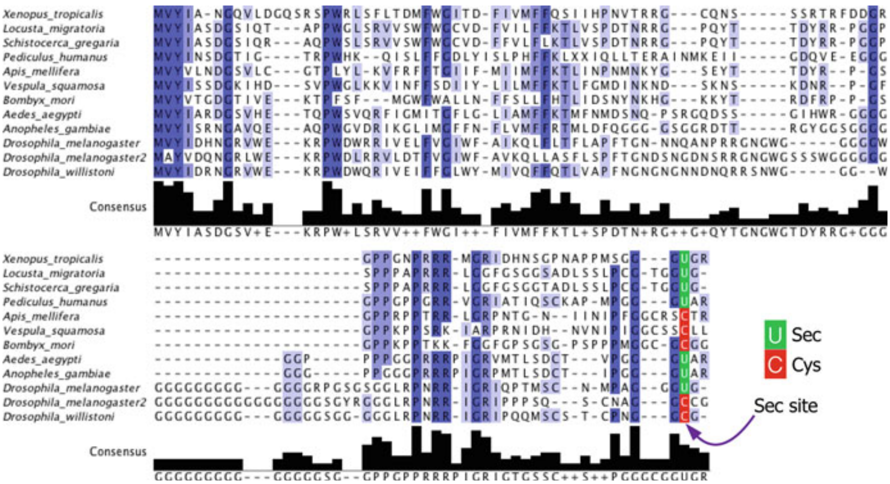


Fig. 5.6 Alignment of SelK protein sequences. This selenoprotein was converted to a Cys homologue in several insects. The Cys paralogue in *D. melanogaster* (SelK2) is also shown

SelK (also called SelG, G-rich, or dSelM, flybase id FBgn0030350) is one of the most widespread selenoproteins in eukaryotes [62]. Although very divergent in sequence among metazoans, its domain structure is conserved and similar to selenoprotein SelS (missing in insects) [88, 96]. SelK is a membrane protein, with Sec found on a glycine-rich cytosolic tail, very close to the C-terminus (Fig. 5.6). In *D. melanogaster* SelK, Sec is the penultimate residue. Its function in this protein is not yet understood. SelK localizes mainly to the ER [96–98], but it is also reported in the plasma membrane [70] and in the Golgi [99]. Its molecular function is still unknown, and there is evidence linking it to very diverse pathways. In mice, SelK overexpression experiments initially led to the proposal that it serves as an antioxidant in cardiomyocytes [97]. Knockout experiments and other evidence support its involvement in immune response and inflammation [100–102]. Recently, immunoprecipitation assays showed that SelK (like SelS) coprecipitates with Derlins and other components of the ERAD complex, suggesting a role in the disposal of glycosylated misfolded proteins [88]. In a study on *D. melanogaster*, SelK knockdown decreased viability and impaired embryonic development, but with no significant change in antioxidant status [93]. A *SelK* paralogue named *SelK2* (or *SelG2*) is present in *D. melanogaster*, with Cys in place of Sec (Fig. 5.6). Among sequenced *Drosophila* genomes, *SelK2* is found only in those belonging to the *melanogaster* group.

SPS2 (also called SEPHS2, flybase id FBgn0032224) was the first selenoprotein identified in fruit fly [103]. SPS2 is responsible for the production of selenophosphate, required for the biosynthesis of selenocysteine. Thus, it is both a selenoprotein and a Sec machinery gene. All other Sec machinery genes are also found in *D. melanogaster*: *tRNAsec* (FBgn0011987), *PSTK* (FBgn0031041), *SecS* (FBgn0037347), *SBP2* (FBgn0087039), *EFsec* (FBgn0034627), and *SECp43*

(FBgn0031607). Surprisingly, impairment of the Sec pathway has no evident effect in *D. melanogaster.* EFsec knockout flies are viable and show no alteration in lifespan or response to oxidative stress [104]. Similarly, a genome-wide screen by RNAi included SPS2 among its targets and reported no evident phenotype in viability [105]. Since Sec machinery is necessary for the expression of selenoproteins, this is in apparent contradiction to the phenotypes reported for SelH and SelK knockdown [93]. A possible explanation is that the phenotypes in [93] were artifactual, caused by off-target effects (possibly on the paralogous genes) [104]. Otherwise, a natural regulatory system may be activated in the absence of Sec machinery, compensating selenoprotein functions and masking clear phenotypes.

5.5.2 **D. willistoni***: The First Selenoproteinless Animal Discovered*

After *D. melanogaster*, the genomes of 11 more *Drosophila* species were sequenced and released in 2007. Among them, a single species was noted as devoid of selenoprotein genes, *D. willistoni* [106]. This was the first documented case of an animal lacking selenoproteins. The *SelH* and *SelK* genes are present in the *D. willistoni* genome, but do not code for selenoproteins. They were converted to Cys homologues (see SelK in Fig. 5.6), whereby Sec UGA mutated to UGU, and the SECIS elements in the 3'UTR degenerated. Phylogenetic analysis clearly ruled out the possible confounding factor of paralogous genes. The Sec to Cys conversion suggests that *SelH* and *SelK* maintained their original function. In contrast, the *SPS2* gene is missing from the genome of *D. willistoni*. Consistently, this species lacks other Sec machinery genes: *tRNAsec*, *PSTK*, *SecS*, and *EFsec*. A scrupulous analysis [74] excluded that the genes were missed because of an incomplete genome assembly, exploiting the synteny with *D. melanogaster*. A degenerated relic of *PSTK* was found in this way. The genes encoding SECp43 and SBP2 proteins were found intact in *D. willistoni*. However, SBP2 presents a single amino acid insertion in the RNA-binding domain, which is predicted to impair its SECIS-binding capacity [74]. Therefore, not only does *D. willistoni* possess no Sec-containing genes, but it also lacks the capacity to code for Sec. In other words, it has lost the Sec trait.

In contrast, all other sequenced *Drosophila* species were found to possess the complete Sec machinery and the same three selenoprotein genes of *D. melanogaster*, with two (possible) exceptions [74]. *D. grimshawi* has an additional selenoprotein, generated by a recent duplication of SelH. In *D. persimilis*, the *SelK* gene displays a single base insertion near the end of the coding sequence. This sets the Sec UGA out of frame, adding nine codons downstream to the next stop (UAA). Additionally, another single-base insertion is found at the core of the SECIS element of *SelK*, which would severely impair its function. For these reasons, SelK may not be a selenoprotein in *D. persimilis.* However, recent data has led us to question this, since this genome assembly may contain many artifactual insertions (unpublished data).

Since 2008, additional *Drosophila* species have been sequenced, and their genomes made public (see http://flybase.org/): *D. biarmipes*, *D. bipectinata*, *D. elegans*, *D. eugracilis*, *D. ficusphila*, *D. kikkawai*, *D. rhopalia*, *D. santomea*, and *D. takahashii*. All of them appear to possess the same selenoproteins and Sec machinery genes of *D. melanogaster*, within some margin of error to consider the quality and completeness of genome assemblies (unpublished results). Thus, the case of *D. willistoni* remains unique among *Drosophila* species.

5.5.3 Selenoproteins in Endopterygota

After *Drosophila*, the first other dipteran species to be sequenced were two mosquitoes, *Anopheles gambiae* [107] and *Aedes aegypti* [108], both vectors of infectious diseases. These two species possess the same three selenoprotein genes found in *D. melanogaster* [30a, 74] (Fig. 5.7). Sec machinery is also present, with the single puzzling exception of *SECp43* missing in *A. gambiae* [74]. This observation is now also supported by the genome analysis of several other recently sequenced *Anopheles* species (Santesmasses, pers. comm.). Since selenoproteins are present and conserved in *Anopheles*, certainly the Sec pathway is functional. Thus, we must assume that the function of *SECp43* has been replaced or somehow became unnecessary in these organisms. In every other aspect, Sec machinery and selenoproteins appear to be homogeneously present in the order of Diptera, with *D. willistoni* being the only known exception.

In contrast, every other sequenced holometabolic insect (superorder Endopterygota) seems to lack the Sec trait (Fig. 5.7). The genomes of silkworm, *Bombyx mori* [109] (order Lepidoptera), and of beetle, *Tribolium castaneum* [110] (order Coleoptera), both revealed the lack of genes for tRNAsec, PSTK, SecS, SBP2, EFsec, and also

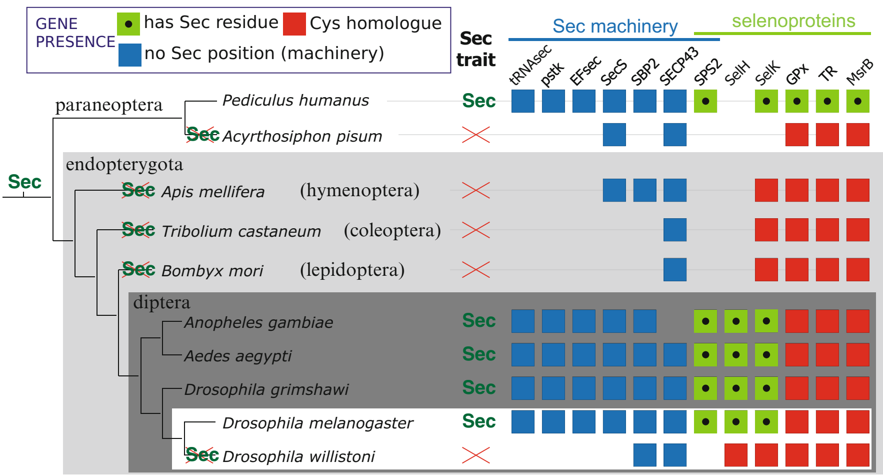


Fig. 5.7 Independent Sec extinctions in insects (Data from [30, 74–76])

SPS2, although a candidate for SECp43 was found [74]. In both species, no detectable homologue could be found for SelH, and only a Cys homologue for SelK was reported [30a]. The search for selenoproteins belonging to other eukaryotic Sec families has also yielded no results [30a, 74].

The honeybee, *A. mellifera* (order Hymenoptera), was also sequenced [111], and its genome searched for selenoproteins and related genes [30a, 74]. Likewise, this insect appears to have lost the Sec trait; tRNAsec, PSTK, EFsec, and also SPS2 are missing (Fig. 5.7). Genes for SecS, SECp43, and SBP2 are still present, arguably to carry out functions now unrelated to Sec. No selenoprotein gene can be found in *A. mellifera*; however, the singular features of the hymenopteran *SPS1* gene have to be mentioned here. As noted earlier, SPS1 is a paralogue of SPS2 present in *D. melanogaster* and many other metazoans. Although similar to SPS2 in sequence, SPS1 does not synthesize selenophosphate and is believed to function in a pathway unrelated to Sec biosynthesis. Part of the reason for this thinking is that SPS1 is conserved in all insect genomes, including those devoid of selenoproteins [30a]. *D. melanogaster* SPS1 contains an arginine aligned to SPS2 Sec. Intriguingly, the *SPS1* gene in *A. mellifera* contains an in-frame UGA, orthologous to the Sec UGA of *SPS2*. This makes it easily mistaken for a selenoprotein gene (as in [30a]). However, the lack of a downstream SECIS element and, most importantly, the lack of essential components of Sec machinery in this genome, strongly argue for a different explanation. We believe that the UGA in *A. mellifera SPS1* undergoes translational readthrough with a mechanism that does not involve Sec insertion [74]. The presence and conservation of UGA at this position can be explained by reason of the phylogenetic history of this gene, recently described in Mariotti et al. [30a].

In recent years, many other insect genomes have been made available. We have searched new genomes for selenoproteins and Sec machinery regularly. So far, our analysis confirms that insects from the orders of Lepidoptera, Coleoptera, and Hymenoptera lack selenoproteins (unpublished results). Besides, in each genome, we detected the same residual Sec machinery genes reported for the model species first analyzed for that insect order: *B. mori* for Lepidoptera, *T. castaneum* for Coleoptera, and *A. mellifera* for Hymenoptera. The only exceptions are *SBP2* and *SecS*, conserved in honeybee but lost in other hymenopterans. Thus, we expect that the most important events regarding selenoproteins and Sec machinery occurred early in the radiation of each of these taxonomic orders.

5.5.4 Selenoproteins in Other Insects

The great majority of available insect sequences belong to the superorder of Endopterygota, which includes all aforementioned orders. However, some genomes from other insect lineages were also recently published. When the pea aphid, *Acyrthosiphon pisum* (superorder Paraneoptera, order Hemiptera), was sequenced, it was noticed that this species had lost the Sec trait [75] (Fig. 5.7). All selenoprotein genes (including *SPS2*) were missing, and Sec machinery genes *tRNAsec*, *PSTK*, *SBP2*, *EFsec* were also absent. *SECp43* and *SecS* are the only Sec machinery

residuals in this genome. In this case, however, the Sec loss did not affect the entire insect order. In fact, selenoproteins were found in other hemipteran insects, as well as in other paraneopterans such as the louse, *Pediculus humanus* (order Phthiraptera) [74]. Importantly, *P. humanus* has selenoproteins belonging to some of the major metazoan Sec families, GPx, TR, and MsrB (Fig. 5.7). The analysis of partial sequences (ESTs) also revealed the presence of selenoproteins in additional non-holometabolic insects, such as grasshoppers and crickets (order Orthoptera) [74].

5.5.5 Independent Sec Extinctions

We have observed that many insect lineages do not possess selenoproteins or the Sec trait. Apart from *A. pisum*, all other known selenoproteinless species are Endopterygota. Here, Diptera constitute the only known Sec-containing lineage, *D. willistoni*, being an isolated exception within the order. Importantly, the phylogenetic structure of selenoproteinless insects (Fig. 5.7) cannot be explained by a single event of *Sec extinction* (loss of Sec trait). Because of its complexity, the reacquisition of Sec machinery through horizontal transfer after a loss is expected to be extremely unlikely in eukaryotes. Also, if this did happen, it would leave a clear phylogenetic signal in Sec machinery genes, which is absent. Instead, the most likely scenario is the continuity of the Sec trait throughout the ancestry of *D. melanogaster*, with a minimum of three selenoproteins in the first Endopterygota insect and additional ones (including GPx, TR, and MsrB) in the first insect. We must conclude that at least five independent Sec extinctions occurred (Fig. 5.7): in Hymenoptera, in Coleoptera, in Lepidoptera, in *A. pisum* (Hemiptera), and in *D. willistoni* (Diptera).

Following each Sec extinction, different Sec machinery genes were maintained in the various lineages. *tRNAsec*, *PSTK*, *EFsec*, and *SPS2* were always lost, indicating roles confined to the Sec pathway. Instead, *SBP2* and *SecS* were kept only by certain phylogenetically scattered lineages. This may denote that these genes were readapted to another function. Lastly, *SECp43* was maintained in all selenoproteinless insects, while it was lost in *Anopheles* (Sec containing). Using the same reasoning previously applied for SPS1 [30a], we think this suggests that insect SECp43 has a function unrelated to Sec. This would imply distinct roles for this protein in insects and vertebrates.

5.5.6 Relaxation of Selective Constraints

Sec losses in insects (and particularly Endopterygota) occurred in parallel lineages after their split. This intriguing pattern can be seen as an example of convergent evolution through loss of function. To justify this process, it was hypothesized that a relaxation in the selective constraints to maintain selenoproteins occurred in insects [74], allowing (or promoting) subsequent losses. This marks a clear difference in the tight conservation of selenoprotein genes in vertebrates and other animals [72]. Since

so many metazoan selenoproteins are involved in oxidative defense, it is natural to search for a rationale in these pathways. Indeed, studies on the antioxidant systems of various insects highlighted fundamental differences with vertebrates. The glutathione reductase family is absent in *D. melanogaster*, and reduction of glutathione appears to be carried out by the TR enzyme instead [112]. On the other hand, Cys homologues of the GPx family are present in fruit fly, but it was shown experimentally that at least one of them uses thioredoxin rather than glutathione as electron donor [113]. Incidentally, this illustrates the inherent limitation of purely bioinformatic approaches to study the evolution of these redox systems; without direct biochemical assays, this important change of substrate would have gone unnoticed. A lot of work remains in characterizing the peculiarities in the antioxidant systems of the insects and in mapping these changes to their phylogenetic tree. Yet, it is clear that major differences exist in the glutathione and thioredoxin systems.

An evident difference concerns the major antioxidant selenoprotein families, TR, GPx, and Msr. These are not present in Sec forms in any Endopterygota, in contrast to the paraneopteran insect, *P. humanus* (Fig. 5.7), and also to other animals including the arthropod, *S. maritima* (Fig. 5.5). It is likely that these Sec families were converted to Cys before the split of Endopterygota. Plausibly, uncoupling these important functions from the Sec pathway should have lowered the selective constraints to further maintain the Sec trait. Previously (approximately after the split with the rest of arthropods), insects had experienced another major selenoproteome reduction with several Sec families lost or converted to Cys (e.g., MsrA, Sel15, SelO, SelS; see Fig. 5.5). In perspective, it is clear that Sec extinctions are the epilogue of a prolonged genomic process, which developed in various steps of selenoprotein depletion.

Many questions still remain. What were the genomic mechanisms by which Sec extinctions occurred? How could these Sec enzymes be converted to Cys despite an expected decrease in catalytic efficiency? Was this process driven by selection or just a series of "frozen accidents?" Did the environment play any role in this instance of convergent evolution? Hopefully, future research will provide some answers.

5.6 A Similar Trend in Nematodes?

Like insects and unlike other animals, nematodes are characterized by a low number of selenoproteins. The genomes of *Caenorhabditis elegans* and *C. briggsae* were thoroughly scanned using three different approaches to find selenoprotein genes [114]. The authors came to the conclusion that a single selenoprotein gene is present, *trxr-1* (TR family). All Sec machinery genes were found in *C. elegans*: *tRNAsec*, *PSTK*, *SecS*, *EFsec*, *SECp43*, and *SBP2*. SPS2 is present in Cys form (very unusual for metazoans), while SPS1 is absent. Identification of SBP2 has been particularly elusive and was only recently accomplished [115]. This protein is much shorter in nematodes compared to insects and other animals and lacks domains that were shown essential for Sec insertion in other species.

It is intriguing that in *Caenorhabditis* the entire Sec machinery apparatus is conserved to recode a single Sec codon. One would expect that the only remaining selenoprotein has an essential function, but this is not the case. Knockout of trxr-1 gives no evident phenotype, unless the gsr-1 encoding gene (glutathione reductase family) is also knocked out [116]. Besides, the authors showed that trxr-1 does not work as thioredoxin reductase in antioxidant defense; instead, it is involved in the removal of old cuticle during molting, a process requiring the reduction of disulfide groups in cuticle components [116]. With a single disposable selenoprotein left, *C. elegans* is expected to be "on the edge" of a selenoprotein extinction, even more than *D. melanogaster*. Yet no Sec extinction events were reported in nematodes until very recently, when it was shown that some plant parasite nematodes (order Tylenchina) have lost the Sec trait [115]. The gene for trxr-1 is absent in these genomes, implying that it was lost (rather than converted to Cys) and suggesting that its function is now carried out by gsr-1. All Sec machinery genes were absent with the only exception of *SECp43*, suggesting again its involvement in a non-Sec-related pathway.

Inspecting the selenoproteins detected in various nematode lineages [114], it appears that all species have a very limited amount. Still, some nematodes possess Sec enzymes for families other than TR; GPx, SPS2, Sel15, SelK, SelT, and SelW were all detected in one or more organisms. The most ancient nematode lineages (e.g., *Xiphinema*, *Trichinella*) are those with most selenoproteins. Thus, it appears that nematodes went through a process of selenoproteome reduction similar to insects, which also culminated in a complete Sec extinction, at least in Tylenchina. Yet, it is clear that the two phenomena in insects and nematodes were phylogenetically distinct events, occurring after the split of the two lineages (Fig. 5.5). How much they have in common remains an open question.

5.7 Concluding Remarks

Sec recoding constitutes an expansion of the genetic code to insert a special amino acid in key sites of certain enzymes. This is a very ancestral feature. Its continuity through the whole tree of life could be disputed, based on the presence of frequent horizontal gene transfer in prokaryotes. However, such continuity in metazoans is unmistakable. This trait was present in the last common ancestor of all animals. It was maintained in the majority of cases, resulting in selenoproteomes of 20 or more genes in extant vertebrates like *Homo sapiens* and also in noninsect arthropods like *S. maritima*. In these organisms, selenoproteins cover many essential roles, particularly in antioxidant defense. In insects, Sec has lost its importance. The selenoproteome gradually decreased in size, either by gene losses or Sec-to-Cys conversions. The process was completed along several parallel lineages resulting in full losses of Sec recoding ability. Apparently, some nematodes endured a similar but independent process.

The history of Sec in these lineages provides a snapshot of evolution at work. It also shows how the recent wealth of publicly available genomes allows us to follow evolutionary processes at this scale. Once the key factors have been elucidated through experiments in model organisms, comparative genomics is a valuable tool to understand functional pathways across lineages. This perspective can also help to highlight important changes, such as different functions for some factors in certain species (like for SPS1 and SECp43, probably unrelated to Sec, at least in insects). In this era of massive sequencing, it is very important to understand how to use observations at the level of genomes to infer gene histories and functional links. In the future, it is likely that automated learning methods will be able to extract such information to predict functional networks. Besides its immediate value for the selenium community, hopefully the history of Sec in insects will also be useful in this context as a prototypical example of a convergent loss of function.

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Chapter 6
Lepidopteran Antimicrobial Peptides (AMPs): Overview, Regulation, Modes of Action, and Therapeutic Potentials of Insect-Derived AMPs

Chandan Badapanda and Surendra K. Chikara

Abstract Due to overuse of antibiotics or drugs, antibiotic resistance and multidrug resistance in pathogenic bacteria have been rising during the last two decades. Therefore, there is an urgent need for development of natural antibiotics in the form of antimicrobial peptides (AMPs). These are produced by nearly all organisms, from bacteria to plants and animals, and can protect against a broad array of pathogenic bacteria, fungi, parasites, viruses, and protozoa and thus may serve as alternatives to synthetic (conventional) antibiotics. Apart from microbicidal properties of AMPs, they have been shown to act as immunomodulators with chemoattractants, to exhibit signaling activities, and also to help in the management of beneficial endosymbionts. As a result, clinical programs on host defense AMPs have been established in the areas of cancer biology, infection, inflammation, and dermatology. This chapter provides a detailed account of the anti-infective properties of AMPs derived from lepidopterans for clinical research in the biotechnology industry. It focuses on the expression of AMPs in insects belonging to the order Lepidoptera upon lipopolysaccharide (LPS) challenge and discusses their various functions and relative mechanisms of action with bacterial membranes. Insect AMPs show greater evolutionary dynamism than conserved components such as signaling molecules, and this may explain insects' ability to mount a high immune response and/or colonize new niches. Thus, understanding the modes of action of these AMPs will give insights into host-parasite coevolution as well as enable design of next-generation antibiotics.

Keywords AMPs • Lepidoptera • Insects • Immunity-related genes • Bioinformatics • Antibiotics

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Abbreviations

AMP	Antimicrobial peptide
CS-αβ motif	Six conserved cysteine residues
DAP	Diaminopimelic acid
DBM	Diamondback moth
EST	Expressed sequence tag
HP-8	Hemolymph proteinase-8
JAK/STAT	Janus kinase/signal transducer and activator of transcription
LPS-K12	Lys-type peptidoglycan from *E. coli* K12
LPS	Lipopolysaccharide
LTA-BS	Lipoteichoic acid from *B. subtilis*
LTA	Lipoteichoic acid
MIN	Minimal inhibitory concentration
MRSA	Methicillin-resistant *S. aureus*
NAG	N-acetylglucosamine
NAM	N-acetylmuramic acid
NGS	Next-generation sequence
PAMP	Pathogen-associated molecular pattern
PG-SA	Lys-type peptidoglycan from *S. aureus*
PG	Peptidoglycan
PGRP	Pathogen recognition protein
RNAi	RNA interference
Spz	Spätzle

6.1 Introduction

The insect order Lepidoptera (butterflies and moths) is the second largest order after the order Coleoptera, with an estimated total of more than 160,000 species falling into more than 130 families [1]. Insects have been remarkably successful in evolution due to their diversity, certainly enhanced by their ability to colonize new niches and feed on nearly all plants and animals, use insects' cells for heterologous protein production in a cost-effective way, and mount a high immune response when faced with a constantly changing and diverse array of pathogens and parasites [2, 3]. To fight against infection or wounding, the insect's immune system employs both cellular and humoral immune systems, although they do not possess adaptive immunity as present in higher vertebrates. Cellular immunity is mainly mediated in hemocytes by executing processes such as phagocytosis, encapsulation, and nodulation [4]. Humoral reactions include synthesis of AMPs which are secreted into the hemolymph and trigger proteolytic cascades leading to coagulation [5], activation of a phenoloxidase cascade to produce the melanin and toxic intermediates against invading pathogens [6], and production of reactive oxygen species [7]. Synthesis of

AMPs in insects occurs in special tissues such as fat bodies (similar to mammalian liver) as well as hemocytes upon microbial infection or septic injury [8]. To date, more than 1750 AMPs have been identified from a variety of organisms; although many are derived from vertebrates and plants, most are derived from invertebrate species [9–14]. The majority of these AMPs have antibacterial properties, followed by a few which are antifungal, anticancer, antiviral, or antiparasitic in nature [10].

Based on amino acid sequence features, AMPs are broadly classified into three categories [15]: first, linear peptides such as cecropin having an α-helix and devoid of cysteine residues; second, peptides with a β-sheet globular structure stabilized by intramolecular disulfide bridges (e.g., heliomicin); and, third, peptides with an overrepresentation of proline or glycine residues (e.g., lebocin and moricin). AMPs are involved in many biological processes such as immune modulation, angiogenesis, and cytokine and histamine release [16–18]. Although AMPs are typically short and show little similarity in sequence, they have some common features such as being highly cationic and tending to form an amphipathic α-helical structure that seems crucial for their function as membrane-active agents or acting on the DNA or RNA inside the cell [19–22]. Compared to cationic AMPs, much less is known about the working mechanism(s) of anionic AMPs [23].

Considering their biological importance, there is a growing need to understand the mechanisms and activities of these molecules. This is greatly facilitated by the availability of genome, transcriptome, and proteome sequences. However, lepidopteran species are relatively underrepresented in terms of genomic resources, and to date genomic sequences from only 5 species are available publicly, including the model silkworm, *Bombyx mori*; the monarch butterfly, *Danaus plexippus*; the Glanville fritillary, *Melitaea cinxia*; the postman butterfly, *Heliconius melpomene*; and the diamondback moth (DBM), *Plutella xylostella* [24–30] (see chapter 3 volume one in this series for more information). Comparative studies of immunity-related genes among lepidopterans and detailed comparative genomics with *B. mori* have been hampered due to a lack of relevant genomic information, but the advent of the next-generation sequence (NGS) platform has facilitated the analysis of innate immunity-related genes from different lepidopterans, such as the tobacco hornworm, *Manduca sexta* [31–33]; the greater wax moth, *Galleria mellonella* [34, 35]; the DBM [36]; the old world bollworm, *Helicoverpa armigera* [37, 38]; the beet armyworm, *Spodoptera exigua* [39]; and the corn earworm, *Heliothis virescens* [40]. Along with NGS, small-scale expressed sequence tag (EST) projects have been performed on many lepidopteran species, for example, the fall armyworm, *Spodoptera frugiperda* [41–44]; the cabbage looper, *Trichoplusia ni* [45]; the tasar silkworm, *Antheraea mylitta* [46, 47]; and the cecropia moth, *Hyalophora cecropia* [48–55] (see chapter 3 volume one in this series for more information).

In this chapter the activities and expression patterns of AMPs upon immune challenge with different microbes are discussed along with their therapeutic potential for the biotechnology industry. A workflow for the identification of AMPs via a comprehensive literature review using different search engines and different database surveys is depicted in Fig. 6.1.

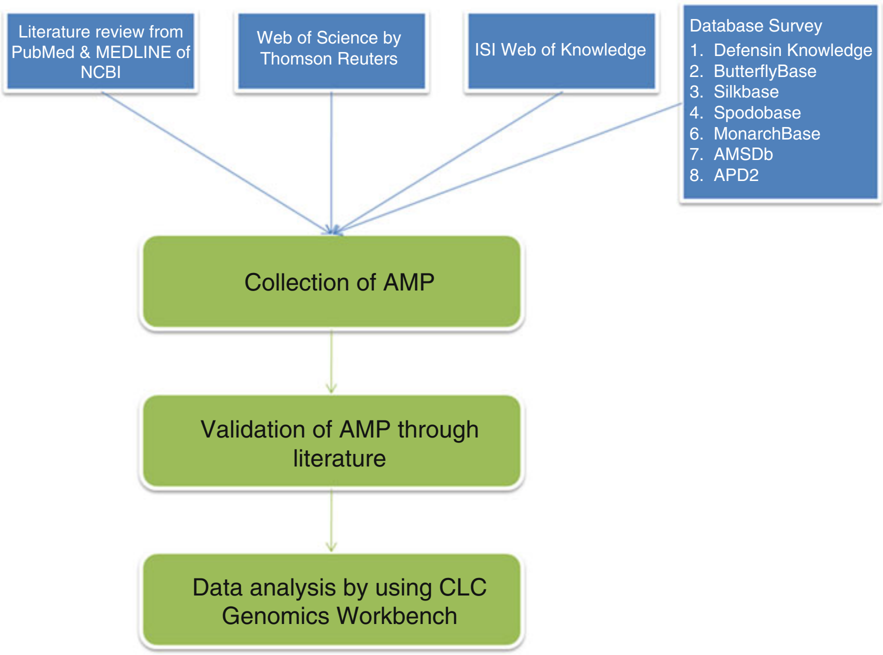


Fig. 6.1 Workflow for the identification of antimicrobial peptides. A comprehensive literature search was conducted using PubMed and MEDLINE at the National Center for Biotechnology Information, Web of Science provided by Thomson Reuters, and ISI Web of Knowledge, to collect and collate the AMPs from the order Lepidoptera. Along with the literature survey, a database survey was conducted to identify peptides falling across moth/butterfly families. Some of the databases used in the study were Defensin Knowledgebase [http://defensins.bii.a-star.edu.sg/], ButterflyBase (http://www.butterflybase.org), SilkBase (http://silkbase.ab.a.u-tokyo.ac.jp/cgi-bin/index.cgi), SPODOBASE [http://bioweb.ensam.inra.fr/spodobase/], MonarchBase (http://monarchbase.umassmed.edu/), and Antimicrobial Sequences Database [AMSDb, http://www.bbcm.univ.trieste.it/~tossi/amsdb.html], APD2 [10]. Amino acid sequences were aligned by CLC Main Workbench and each visually inspected for regions of high-quality alignment

6.2 Antimicrobial Peptides (AMPs) in Lepidopterans

Antimicrobial peptides in Lepidoptera are grouped into different families, namely, cecropins, attacins, moricins, lebocins, gloverins, defensins, and defensin-like (spodoptericin, heliomicin, gallerimycin, galiomicin) and anionic peptides, lyozymes, cobatoxin, and X-tox. Apart from these, rarely found species-specific AMPs like hinnavins I and II [56, 57]; dipausin [37]; hyphancins 3D, 3E, 3 F, and 3G (accession numbers P50720, P50721, P50722, P50723); and papiliocin [58, 59] are reported in this chapter. Out of all the AMPs found in the order Lepidoptera, the moricins and the moricin-like gene family, the gloverins, and the cecropins are predominantly present and are multigene family proteins. Moricins and gloverins are found to be restricted to the order Lepidoptera, whereas the cecropins are found to be widely distributed in the animal kingdom.

6.2.1 Cecropins

Cecropin was the first and most extensively studied α-helical, cationic, immune-inducible AMP, discovered in the hemolymph of a wild silkmoth (Saturniidae) *H. cecropia* [48]. Cecropins are widespread in their distribution in the animal kingdom as they are found not only in insects but also in tunicates and *Ascaris* nematodes. Mature cecropin peptides are at least 35–39 amino acids in length, lack cysteine residues, and comprise two linear α-helices connected by a hinge through which they disintegrate the bacterial membrane, subsequently targeting intracellular targets inside bacterial cells [60]. At least 11 genes encoding for cecropin are present in the *B. mori* genome and are classified into five subtypes (A–E). Six of these cecropin-encoding genes are classified as cecropin-B type and are clustered on chromosome 26, suggesting that an expansion of this subtype occurred in the silkworm genome by gene duplication [61]. Cecropins are found to exhibit antiviral, antibacterial, and antifungal activities; these are listed in Table 6.1.

6.2.2 Moricins

Moricins and moricin-like gene family members resemble an amphipathic α-helical structure and were first identified in *B. mori* [62]. At least 13 genes encoding for moricins were identified in the *B. mori* genome, of which one encodes moricins, four encode a moricin-like A subtype, and eight encode a moricin-like B subtype [61]. Recently, from NGS analysis of *G. mellonella*, at least 6 contigs encoding moricin-like genes were identified [34]. Moricins act against LPS of bacterial membranes and inhibit formation of the outer membrane. Moricins are found across different lepidopteran species, and their activities against bacteria, fungi, and yeast are listed in Table 6.1.

6.2.3 Defensins

Defensins have been isolated from fungi, plants, insects, mussels, birds, and various mammals. Based on the spacing pattern of cysteines, defensins are broadly divided into five groups: plant, invertebrate, α-, β-, and Θ-defensins, with the last three groups being mostly found in mammalian species [63]. Insect defensins are small (3–5 KDa) cationic peptides which contain six conserved cysteine residues (CS-αβ motif) involved in three disulfide bridges. The 3-dimensional structure of defensin consists of an N-terminal loop followed by mixed α-helix and antiparallel β-sheets [64]. This kind of structure facilitates the positively charged defensins to penetrate into the negatively charged bacterial membrane leading to its disruption [65].

Insect defensins are mainly active against Gram-positive bacteria, but some insect defensins are also active against Gram-negative bacteria and fungi [66]. The

Table 6.1 Antimicrobial peptides and proteins from lepidopterans (moths and butterflies) through a literature-based search

Name (number of peptides)	Characteristics	Activity against	Reference
Bombyx mori (37)			
Cecropin-A (2)	Linear, amphipathic, α-helical	Bacteria	[24]
Cecropin-B (6), cecropin-C (1), cecropin-D (1), cecropin-E (1)	Linear, amphipathic, α-helical, lies in chromosome 26	Bacteria	[24]
Moricins-A (4), moricins-B (8)	Amphipathic, α-helical	Bacteria	[24, 62]
Gloverins-A (3), gloverins-B (1)	Basic, heat stable, glycine rich; Glo-A lies in chromosome 28 and Glo-B lies in chromosome 17	Bacteria	[24, 61]
Attacins (2)	Glycine rich, chromosome 6	Bacteria	[24]
Ebocins (2)	Chromosome 26	Bacteria	[24]
Lebocin (1)	Proline rich	Bacteria	[24]
Defensin (1)	Cysteine rich, chromosome 13	Bacteria, fungi, yeast	[24]
Lysozyme (1) and lysozyme-like (3)	Protein	Bacteria	[24]
Danaus plexippus			
Cecropin-A (5)	Linear, amphipathic, α-helical	Not tested, probable antibacterial activity	[26, 27]
Moricin and moricin-like (3)	Amphipathic, α-helical	Not tested	[26, 27]
Gloverin (1) EHJ66058	Basic, heat stable, glycine rich	Not tested	[26, 27]
Attacin and attacin-like (3)	Glycine rich	Not tested	[26, 27]
Defensin (EHJ63539)	Cysteine	Not tested	[26, 27]
Gallerimycin (EHJ75242.1)	Defensin-like	Not tested, probable antifungal activity	[26, 27]
Proline rich (EHJ64534.1)	Rich in proline residues	Not tested	[26, 27]
I-type lysozyme (4)	Protein	Not tested	[26, 27]
Lysozyme-like (6)	Protein	Not tested	[26, 27]
Putative defense protein Hdd11 (EHJ65746.1)	Putative defense protein	Not tested	[26, 27]
Immune-related Hdd13 (EHJ78998.1)	Putative immune protein	Not tested	[26, 27]
Hyphancin-3E (EHJ64256.1)		Not tested	[26, 27]
Galleria mellonella			
Cecropins	Linear, amphipathic, α-helical	Yeast, bacteria, fungi	[34, 35, 107]
Galiomicin	Similar to heliomicin	Bacteria, filamentous fungi	[34, 35, 107]

(continued)

Table 6.1 (continued)

Name (number of peptides)	Characteristics	Activity against	Reference
Gallerimycin	Defensin-like, 3 disulfide bonds	Yeast, filamentous fungi	[34, 35, 107]
Gloverins	Glycine rich	Bacteria	[34, 35, 107]
Lebocins	Linear, proline rich	Bacteria	[34, 35, 107]
Moricins	Amphipathic, α-helical	Yeast, bacteria, fungi	[34, 35, 107]
Anionic peptide-1 and anionic peptide-2	Linear	Bacteria, fungi	[77, 107]
Proline-rich peptide	Linear, proline rich	Bacteria	[34, 35, 107]
Spodoptericin	Defensin-like	Bacteria	[34, 35, 107]
Lysozymes	Protein	Yeast, bacteria, fungi	[34, 35, 107]
Gal6-tox, x-tox	Atypical defensin	Not known	[34, 35, 107]
IMPI	5 disulfide bonds	Thermolysin inhibitor and metalloproteinase inhibitor	[95, 107]
Helicoverpa armigera			
Cecropin-1 (GU182916), cecropin-3 (GU182910)	Linear, amphipathic, α-helical	Bacteria, fungi, virus	[37, 38]
Cecropin-2 (GU182909)	Linear, amphipathic, α-helical	Bacteria, virus	[37, 38]
Moricin-like (GU182911)	Amphipathic, α-helical	Bacteria, fungi	[37, 38]
Gloverin-like (GU182908)	Basic, heat stable, glycine rich	Bacteria, fungi, virus	[37, 38]
Gallerimycin-like (GU182913)	Defensin-like, 3 disulfide bonds	Gram-negative bacteria, fungi	[37, 38]
Attacin (GU182917)	Glycine rich	Bacteria, fungi	[37, 38]
Galiomicin-like (GU182907)	Similar to heliomicin	Bacteria, fungi, virus	[37, 38]
Lysozyme (GU182915)	Protein	Bacteria, fungi, virus	[37, 38]
Cobatoxin-like (GU182912)	Atypical defensin, cysteine rich	Bacteria, fungi	[37, 38]
Immune inducible protein (DQ875243)	Defense protein	Fungi	[37, 38]
Heliothis virescens			
Cecropin-3, cecropin-A2, cecropin-D	Linear, amphipathic, α-helical	Bacteria and fungi	[40]
Gallerimycin	Defensin-like, 3 disulfide bonds	Bacteria and fungi	[40]
Gloverin-like	Basic, heat stable, glycine rich	Bacteria and fungi	[40]

(continued)

Table 6.1 (continued)

Name (number of peptides)	Characteristics	Activity against	Reference
Attacin-A precursor	Glycine rich	Bacteria and fungi	[40]
Heliocin	Defensin-like	Bacteria and fungi	[40]
3-tox, 5-tox, 6-tox	Atypical defensin	Bacteria and fungi	[40]
Cobatoxin B	Atypical defensin	Bacteria and fungi	[40]
Lysozyme	Protein	Bacteria and fungi	[40]
I-type lysozyme	Protein having 5 to 6 disulfide bonds formed by cysteine residues	Bacteria and fungi	[40]
IMPI	Protein	Bacteria and fungi	[40]
Immune-inducible protein	Defense protein	Bacteria and fungi	[40]
Spodoptera exigua			
Cecropin	Linear, amphipathic, α-helical	Bacteria, yeast, SeMNPV	[39]
Attacin	Glycine rich	Bacteria, yeast, SeMNPV	[39]
Gloverin	Basic, heat stable, glycine rich	Bacteria, yeast, SeMNPV	[39]
Diapausin	N-type voltage-gated calcium channel blocker peptide	Bacteria, yeast, SeMNPV	[39]
Lebocin	Linear, proline rich	Bacteria, yeast, SeMNPV	[39]
Moricin	Amphipathic, α-helical	Bacteria, yeast, SeMNPV	[39]
Cobatoxin	Atypical defensin	Bacteria, yeast, SeMNPV	[39]
Lysozyme	Protein	Bacteria, yeast, SeMNPV	[39]
Plutella xylostella			
Moricin-like peptide C2 (JL943792)	Amphipathic, α-helical	*D. semiclausum* ichnovirus	[36]
Gloverin (JL943753)	Glycine rich	*D. semiclausum* ichnovirus	[36]
Proline rich (JL943855)	Rich in proline residues	*D. semiclausum* ichnovirus	[36]
Cecropin-E (JL943818)	Linear, amphipathic, α-helical	*D. semiclausum* ichnovirus	[36]
Cecropin-1 (JL943850)	Linear, amphipathic, α-helical	*D. semiclausum* ichnovirus	[36]
Lysozyme II (JL943860)	Protein	*D. semiclausum* ichnovirus	[36]
Immune-related Hdd1 (JL943800)	Defense protein	*D. semiclausum* ichnovirus	[36]

(continued)

Table 6.1 (continued)

Name (number of peptides)	Characteristics	Activity against	Reference
Manduca sexta			[31–33]
Cecropin	Linear, amphipathic, α-helical	Bacteria	[31–33]
Moricin	Amphipathic, α-helical	Bacteria	[31–33]
Gloverin	Glycine rich	Bacteria, fungi	[31–33, 87]
Lebocin	Linear, proline rich	Bacteria	[31–33, 75, 76]
Attacin	Glycine rich	Bacteria	[31–33]
Lysozyme	Protein	Bacteriolytic	[31–33]
X-tox	Atypical defensin	Bacteria	[26, 27, 31]
Spodoptera frugiperda			
Cecropin-B1	Linear, amphipathic, α-helical	Bacteria, fungi	[41, 43]
Spodoptericin (AAQ18895)	Defensin-like	Bacteria, fungi	[43, 44]
Gallerimycin (AAP69838)	Defensin-like, 3 internal disulfide bond	Bacteria	[43, 44]
Cobatoxin (AAP69839)	Atypical defensin	Bacteria	[43, 44]
Lysozyme	Protein		[42, 43]
Antheraea mylitta			
Cecropin-like (ABG72697)	Linear, amphipathic, α-helical	Bacteria	[46]
Attacin-like (ABG72693)	Glycine rich	Bacteria	[46]
Gloverin-like (ABG72699)	Basic, heat stable, glycine rich	Bacteria	[46]
Lebocin-like (ABG72703)	Linear, proline rich	Bacteria	[46]
Lysozyme (Q7SID7)	Protein	Bacteria	[47]
Putative defense protein 1(Q0Q029)	Putative secretory defense protein	Bacteria	[46]
Trichoplusia ni			
Cecropin-A (P50724.1)	Linear, amphipathic, α-helical	Bacteria	[45]
Cecropin-B (ABV68872.1)	Linear, amphipathic, α-helical	Bacteria	[45]
Cecropin-D (ABV68873.1)	Linear, amphipathic, α-helical	Bacteria	[45]
Defensin (ABV68852)	3 disulfide bond	Bacteria	[45]
Gallerimycin (ABV68855)	Defensin-like, 3 disulfide bond	Bacteria	[45]
Attacin-A (P50725)	Glycine rich	Bacteria	[45]
Gloverin (ABV68856)	Basic, heat stable, glycine rich	Bacteria	[45]
Cobatoxin-like (ABV68851)	Atypical defensin	Bacteria	[45]

(continued)

Table 6.1 (continued)

Name (number of peptides)	Characteristics	Activity against	Reference
Lebocin (AAG44366)	Linear, proline rich	Bacteria	[45]
Lysozyme (P50718.1)	Protein	Bactericidal	[45]
Hyalophora cecropia			
Cecropin-A (AAA29185.1)	Linear, amphipathic, α-helical	Bacteria	[48, 51, 52]
Cecropin-B (AAA29184.1)	Linear, amphipathic, α-helical	Bacteria	[48, 51, 52]
Cecropin-D (AAA29186.1)	Linear, amphipathic, α-helical	Bacteria	[48, 51, 52]
Attacin-B (P01512.2)	Glycine rich	Bacteria	[49–51, 53]
Attacin-E (P01513.2)	Glycine rich	Bacteria	[49–51, 53]
Gloverin (P81048.2)	Basic, heat stable, glycine rich	Bacteria	[55]
Lysozyme (P05105.2)	Protein	Bacteria	[51, 54]

first lepidopteran defensin was identified in *H. virescens*, an immune-inducible AMP known as heliomicin and found to be active against fungi [67, 68]. Cysteine-rich defensin-like peptides such as gallerimycin, galiomicin, spodoptericin, and cobatoxin [34, 69] have been identified from *G. mellonella*. Both galiomicin and gallerimycin are found to be active against fungi as well as the human pathogenic yeast *Candida albicans* [34, 35].

A defensin-like peptide known as X-tox was identified from *S. frugiperda* and found to have a variable number of CS-αβ motifs [64]. The AMPs belonging to X-tox are restricted to the order Lepidoptera. Recently, it has been reported that X-tox from *S. frugiperda* has lost the antimicrobial properties of the ancestral insect defensins, and thus the X-tox protein family from Lepidoptera will provide a valuable and tractable model to improve the understanding of the molecular evolution of defensins [70]. The defensin CS-αβ motif from insects forms a structural scaffold which resembles that of cobatoxin, a 6-tox protein shown in Fig. 6.2 in multiple sequence alignment with related proteins. The knottin fold is characterized by three disulfide bridges, in which disulfide bridges are formed between six cysteine residues. The six cysteine residues of defensin-like peptides are conserved in insects as well as plants and share similarities with the antifungal peptides, the knottin fold, and the sodium channel toxin protein and share similar structural scaffolds by forming disulfide bridges. A multiple sequence alignment of defensins and defensin-like peptides is shown in Fig. 6.3.

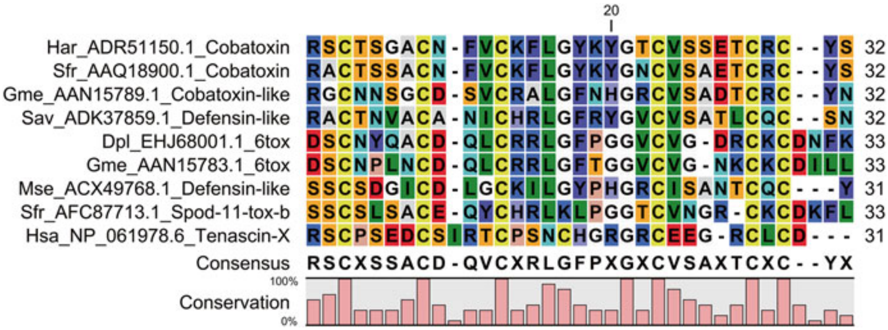


Fig. 6.2 Partial multiple sequence alignments of cobatoxin and related proteins having six conserved cysteine residues forming three disulfide bridges. Tenascin X from human (*Homo sapiens*) also has homology to cobatoxin and defensin-like peptides, showing their evolutionary relationship. Species abbreviations along with accession numbers and gene functions are shown. The full scientific names of the species are as follows: Har (*Helicoverpa armigera*), Sfr (*Spodoptera frugiperda*), Gme (*Galleria mellonella*), Dpl (*Danaus plexippus*), Mse (*Manduca sexta*), and Sav (dipteran, *Sitobion avenae*). Alignments were performed with CLC Workbench

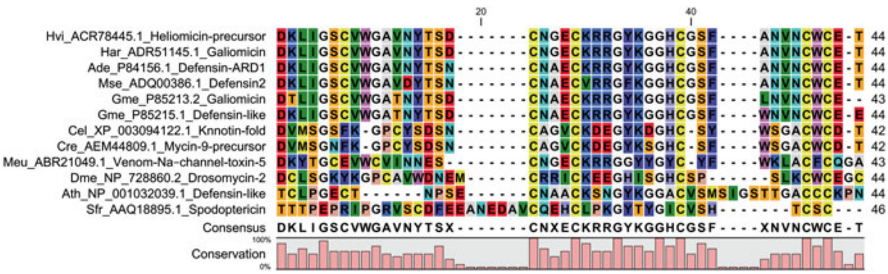


Fig. 6.3 Multiple sequence alignments of mature peptides containing a knottin fold, defensins and defensin-like peptides (heliomicin, galiomicin, spodoptericins, and drosomycin), and antifungal peptides from different insects show their functional diversity. The six cysteine residues of defensin-like peptides are conserved in insects as well as plants and share similarities with the antifungal peptides, the knottin fold, and the sodium channel toxin protein and share similar structural scaffolds by forming disulfide bridges. Species abbreviations, accession numbers, and gene functions are shown. The full scientific names of species are as follows: Hvi (tobacco budworm, *Heliothis virescens*), Har (cotton bollworm, *Helicoverpa armigera*), Ada (one-spotted prepona, *Archaeoprepona demophon*), Mse (tobacco hornworm, *Manduca sexta*), Gme (greater waxmoth, *Galleria mellonella*), Cel (nematode, *Caenorhabditis elegans*), Cre (nematode, *Caenorhabditis remanei*), Meu (lesser Asian scorpion, *Mesobuthus eupeus*), Dme (fruit fly, *Drosophila melanogaster*), Ath (plant, *Arabidopsis thaliana*), and Sfr (fall armyworm, *Spodoptera frugiperda*)

6.2.4 Classification of AMPs Based on Amino Acid Overrepresentation

AMPs are classified by the overrepresentation of particular amino acids in their structure. For example, in Lepidoptera glycine-rich AMPs are classified under attacins or gloverins, and proline-rich AMPs are classified under lebocins. Mostly proline-rich AMPs are active against Gram-negative bacteria. Unlike other AMPs, their mode of action does not involve lysis of the bacterial membrane; rather, they act intracellularly, and for this property these kinds of AMPs can be used as lead anti-infective compounds both in bacteria and eukaryotic cells [71].

6.2.4.1 Attacins

Attacins are glycine-rich bacteriostatic proteins with a size of 20 kDa, first discovered in the wild silkmoth, *H. cecropia*, and shown to interact with LPS of Gram-negative bacteria leading to abnormal cell division and ultimately bacterial cell death [49]. *H. cecropia* has two isoforms of attacins having 80 % sequence identity with each other, but one isoform is acidic (calculated pI 6.0), and the other one is basic (calculated pI, 9.1). Attacin from *H. cecropia* shares homology with the Attacin-A gene from *Drosophila melanogaster* and sarcotoxin from *Sarcophaga peregrina*, which also shares the property of glycine-rich residues [72]. Treatment of *Escherichia coli* with attacin leads to an increase in outer membrane permeability. Inhibition of outer membrane protein synthesis is achieved on the transcriptional level and triggered by binding of attacin to the cell surface without entering the inner membrane or cytoplasm. Primary binding occurs on LPS, explaining why the basic attacin is more active against *E. coli* than the acidic form [50].

6.2.4.2 Gloverins

Gloverin was first identified from the wild silkmoth, *H. gloveri*, and found to be a basic, heat stable, glycine-rich AMP lacking cysteine residues [73]. In all at least four genes encoding gloverins were identified from the genome of *B. mori*, out of which gloverins 2, 3, and 4 are clustered on chromosome 28 and found to be derived from the ancestral gloverin 1 by gene duplication events. All four gloverins are found to be active against *E. coli*, but expression of gloverin 1 is not observed in the embryonic stage, whereas gloverins 2, 3, and 4 are found to be expressed in all embryonic stages [61, 73]. At least five contigs representing gloverins were identified from the NGS analysis of *G. mellonella* and found to be putative antibacterial peptides. Expression of gloverin mRNA in *M. sexta* larvae is upregulated by peptidoglycans (PGs) of *E. coli* and *Staphylococcus aureus* and found to be regulated by the Toll signaling pathway [74].

6.2.4.3 Lebocins

Lebocin was first reported as a differentially expressed gene in response to bacterial infection in a subtractive suppression hybridization experiment in *M. sexta* [75]. It was hypothesized that there should be a common mechanism to generate active lebocin peptides from a polypeptide precursor, and this hypothesis was supported after the identification of lebocin from the hemolymph of G. *mellonella*, *H. armigera*, *B. mori*, and *M. sexta* [76]. Mostly lebocins are found to be active against bacteria and are upregulated upon immune challenge with them. The functions of lebocins from the lepidopterans are listed in Table 6.1.

6.2.5 Anionic Peptides

Anionic AMPs have been established as a vital component of the innate immune systems of vertebrates, plants, and invertebrates and found to be active against bacteria, fungi, and viruses. Anionic peptides generally adopt amphiphilic structures, but the exact mechanism and modes of action of these peptides are not well studied to date [23]. In *G. mellonella* two anionic peptides have been identified. Anionic peptide 1 (P85211) is proline rich and shows potent inhibitor activity against the Gram-positive bacterium *Micrococcus luteus*; it also inhibits fungi at greater concentrations. Anionic peptide 2 (P85216) has activity against the yeast, *Pichia pastoris*, but activity against Gram-positive bacteria is found only at a high concentration [35, 77]. In multiple sequence alignments with anionic peptide 1 from *G. mellonella*, significant matches were established with lebocin 3 and 4 precursors of *B. mori*, as well as with other lepidopteran lebocin-like sequences; by contrast, using anionic peptide 2, no hit was found matching with any antimicrobial peptides from other species (unpublished).

6.2.6 Lysozymes

6.2.6.1 C-Type Lysozymes (Chicken-Type Lysozymes)

Lysozymes (muramidases) exhibit catalytic activity by cleaving the β (1, 4)-glycosidic bond between the N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG) residues of bacterial peptidoglycan. Reports suggest that lysozymes actively target the peptidoglycans from Gram-positive bacteria but not those of Gram-negative bacteria, as the latter are not directly accessible by lysozymes because of the presence of lipopolysaccharides in their outer membrane. Only a few studies have been reported on the antibacterial properties from arthropod C-type lysozymes against Gram-negative bacteria [42, 78, 79]. Also, it can be noted that lysozyme-like sequences sometimes lack muramidase activities, although they share sequence identity with lysozyme, for example, in *B. mori* [80]. The activities tested for lysozymes are shown in Table 6.1.

6.2.6.2 I-Type Lysozymes (Invertebrate-Type Lysozymes)

Lysozymes found in invertebrates are designated as I-type lysozymes and are absent from vertebrate genomes. The invertebrate I-type lysozymes, although somewhat diverged in their activities, have 10 or 12 cysteine residues forming five or six disulfide bonds, possibly giving stability to their structure [34, 26, 27]. In addition, from genomic sequence comparisons, it is inferred that I-type lysozyme homologues are widespread in Lepidoptera, if not universally present in insects.

In summary, defensins play a prominent role in the anti-Gram-positive response of coleopterans, dipterans, and possibly hymenopterans, while lepidopterans rely on cecropins and lysozymes. Attacins and coleoptericins work in a similar fashion not by forming pores in the membrane of bacteria but by forming chains, subsequently affecting the bacterial cell cycle. Unlike these two AMPs, cecropin works in a different way, by penetrating the bacterial cell wall and other intracellular targets inside bacterial cells.

Gene duplication is the primary source of obtaining new genes with novel or altered function during the evolution of a species. From this review, it is observed that significant gene expansion has occurred for cecropins (at least 11 genes) and moricins (at least 12 genes) of *B. mori*, lysozymes or lysozyme-like sequences from *D. plexippus* (at least 10 genes), and hyphancins from fall webworm *Hyphantria cunea* (at least 4 genes). In conclusion, lepidopterans rely in a major way on cecropins and lysozymes, which show even greater functional diversification than other AMPs described in this chapter. This functional diversification may explain lepidopterans' ability to mount a high immune response and potentially increase or enhance their ability to colonize new niches.

6.3 Regulation of Lepidopteran AMPs

Infection-induced transcriptional regulation of AMPs is achieved by at least three main signaling pathways in Lepidoptera: the Toll pathway [81, 82], the immune deficiency (Imd) pathway [83], and the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathways [39, 84]. The Toll pathway is activated by its ligand Spätzle (Spz) and has been identified in many insects [85, 86]; direct evidence for a Toll-Spz pathway regulating the expression of AMP genes, including cecropin-6, attacin1, attacin2, moricin, and lebocin, was demonstrated recently through an in vivo assay in *M. sexta* [87]. ProSpätzle is synthesized as an inactive precursor which is secreted into the hemolymph and converted into its active form (Spz) by a proteolytic processing enzyme (SPE) to act with the Toll ligand. Hemolymph proteinase-8 (HP-8) is a plasma zymogen present in *M. sexta* which is an apparent orthologue of the easter and Spz enzymes of *D. melanogaster*. Injection of HP-8 into *M. sexta* larvae stimulates the expression of attacin, cecropin, gloverin, and moricin and elevates plasma antibacterial activity. ProHP-8 is the inactive form of HP-8 and is activated by the CLIP-domain proteinase HP-6 (similar to

orthologues of *persephone* in *Drosophila*); injection of recombinant HP-6 also induces the expression of AMP in *M. sexta* larvae [88].

The mechanism of signaling and AMP induction in the lepidopteran, *M. sexta*, and the dipteran, *D. melanogaster*, is illustrated in Fig. 6.4. In *D. melanogaster*, AMP expression is mainly regulated by the Toll and the Imd pathways upon challenge with bacterial LPS [89]. In *M. sexta*, LPS, a major component of Gram-negative bacteria, and lipoteichoic acid (LTA), a major component of Gram-positive bacteria, are both used as elicitors for the activation of AMPs. Experimental results indicate that the *M. sexta* Toll pathway may be activated significantly by Lys-type peptidoglycan from *S. aureus* (PG-SA), LPS from *E. coli* K12 (LPS-K12), and LTA from *B. subtilis* (LTA-BS). In contrast, diaminopimelic acid (DAP) PG-K12 from *E. coli*, PG-BS from *B. subtilis* (DAP-type PGs), and LTA-SA from *S. aureus* may play roles in the Imd pathway [90]. This is the first report describing different components of peptidoglycan, especially LTA, as bacterial elicitors that stimulate AMP expression in a lepidopteran. But there are limitations to all of the above signaling

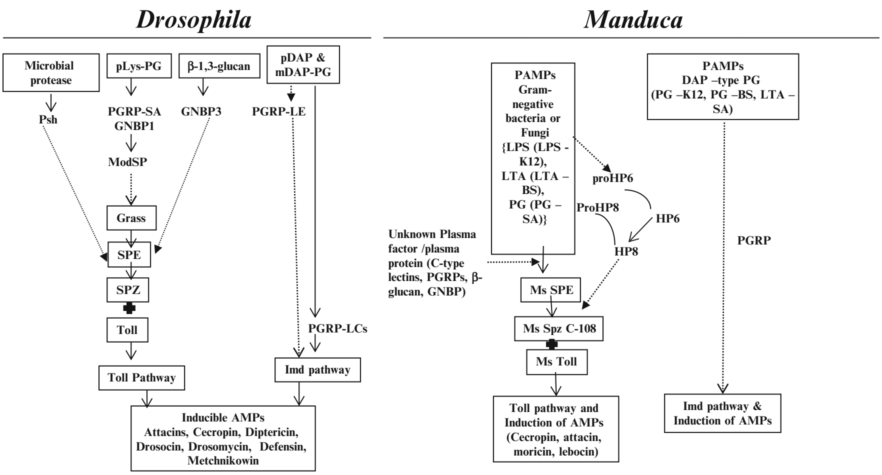


Fig. 6.4 Comparison of the activation mechanism of AMPs after recognition of microbial molecules in the lepidopteran, tobacco hornworm, *Manduca sexta*, and the dipteran, *Drosophila melanogaster*. Drosomycin and diptericin are mainly produced after activation of the Toll and Imd pathways in *Drosophila*. The other five *Drosophila* AMPs are known to be produced by a synergistic interaction between these two pathways [82]. The first direct experimental evidence for a *Toll-Spz* pathway regulating the expression of AMP genes in a lepidopteran, *M. sexta*, was demonstrated recently [87], as shown in the figure. Also depicted are the signaling elements of the immune pathways in tobacco hornworm and flies. It is inferred that the AMP recognition pathways in flies are well defined, whereas identification of the pathways in tobacco hornworm is still in progress. *Dashed arrows* indicate steps that have not been experimentally verified or for which components of the pathway have not been identified. HP-6 or 8, hemolymph proteinase-6 or 8; PGRP, peptidoglycan recognition protein; GNBP, Gram-negative bacteria binding protein; SPZ, Spätzle; pLys-PG, polymeric lysine-type peptidoglycan; pDAP-PG, polymeric meso-diaminopimelic acid-type peptidoglycan; Psh, persephone; mDAP-PG, monomeric meso-diaminopimelic acid-type peptidoglycan; PAMPs, pathogen-associated molecular patterns; Ms, *Manduca sexta*

studies because so far there is less knowledge of the structures of pathogen recognition proteins (PGRPs) in Lepidoptera compared to *D. melanogaster*. Although 12 genes encode PGRP in *B. mori*, exactly which PGRP binds to which pathogen-associated molecular pattern (PAMP) in the Toll/Imd pathway is yet to be established. In contrast, due to rapid progress in RNA interference (RNAi) studies in insects, our understanding of the AMPs and their regulatory pathways is likely to increase significantly in the near future.

6.4 Therapeutic Potential of Lepidopteran AMPs

The disadvantages of research on the therapeutic potential of AMPs are their high cost, limited stability, and unknown toxicity and pharmacokinetics. Reabsorption of orally administered AMPs into the systemic circulation may be prevented as they may be degraded before reaching the gastrointestinal tract [91, 92]. AMPs occur at very low concentrations naturally, and the cost of peptide production by solid-phase synthesis is very expensive. But the cost can be reduced and production can be enhanced by using recombinant technology [3, 93].

On the other hand, there are advantages in AMP research both from academic and industrial points of view. Although these peptides have enormous diversity, certain commonalities exist among AMPs, as they share a net positive charge and consist of approximately 50 % hydrophobic residues. These physicochemical properties confer on AMPs the ability to fold into amphiphilic conformation upon interaction with bacterial membranes [94]. In contrast to AMPs produced in bacteria, insect-derived AMPs form correctly folded structures, the secretory AMPs form disulfide bridges for providing structural stability, glycosylation occurs at appropriate sites, and a few AMPs from insect origin are so unique compared to the human proteome that they can elicit a strong antigenic response. Additionally, insect-derived AMPs/or proteins (such as an inducible metalloproteinase inhibitor from *G. mellonella*) can overcome cytotoxicity and susceptibility to proteolysis [95].

Mixed successes are not deterring companies from developing AMPs as anti-infective therapeutics. The trend is to develop smaller length peptides with low molecular weight than the native proteins, which are cheaper to synthesize. The drug hexapeptide-7 derived from cecropin-B by the company Helix BioMedix has reached clinical phase I trials and will be used for wound healing and skin degeneration [96].

Cecropin-AD (a chimeric AMP obtained from cecropin) displays antibacterial activity against both Gram-positive and Gram-negative bacteria and has been recombinantly expressed in *B. subtilis* [93]; this system could be applied as a powerful tool for large-scale production of cecropin-AD. The antibacterial activity is present at temperature and pH ranges commonly found in the animal body and resistant to cleavage by protease enzymes and at low minimal inhibitory concentrations (MICs). These properties make cecropin-AD a useful therapeutic agent for

animals and possibly humans [93]. Cecropins A and B help prevent the proliferation of cancer cells and therefore have potential for the development of anticancer drugs [97, 98].

Heliomicin, as indicated above first identified from *H. virescens*, possesses activity against life-threatening fungal infection in immune-suppressed humans and is at an advanced preclinical stage of development [96]. Furthermore, the antifungal role of many defensins, such as drosomycin, is well known, and their potential for development of antimycotics is under investigation [99]. Gallerimycin is another antifungal peptide, as noted above first identified from greater wax moth *G. mellonella*. Its transgenic expression in plants has been shown to protect them from fungal infection [100], suggesting that it can be used for crop protection.

Major contributions of insect AMPs to medicine can potentially be made as therapeutic agents against antibiotic-resistant bacterial infection. The synthetic 9-mer peptides, ALYLAIRRR-NH2 and ALYLAIRKR-NH2, designed from the amino acid sequences of active sites of insect defensins, were found to protect infected mice from lethal methicillin-resistant *S. aureus* (MRSA) through in vivo study [100]. A synthetic insect defensin has been used to treat silk sutures and efficiently kill MRSA [91, 101]. And finally, moricins produced by recombinant methods in *E. coli* were found to be active against methicillin-resistant *S. aureus*, suggesting they might also be used as antimicrobial agents in the future [102].

It was observed that hinnavin II from the cabbage butterfly, *Artogeia rapae*, has a powerful synergistic effect on the inhibition of bacterial growth with purified lysozyme [57]. The results of this study suggest that, in addition to the direct modes of action described above, activities of these natural AMPs can be enhanced through a synergistic approach to target different pathogenic bacteria.

Although many insect-derived AMPs were first identified in the Lepidoptera, the success rate for developing new applications has been very limited, and no AMPs have been approved to date for systemic use in humans [103]. Industrial products for external use of AMPs (such as mouthwashes for gingivitis and treating catheter-related and hospital infections) from vertebrate sources are in advanced stage clinical trials and about to enter the market very soon [91, 94]. The idea of using the burying beetle, *Nicrophorus vespilloides*, as a source of natural products with the potential for application in medicine or food preservation is being explored [104]; similarly, AMPs from lepidopterans can be explored for these kinds of biotechnology applications.

Upon infection with bacteria or fungi, *G. mellonella* produces an inducible metalloproteinase inhibitor along with other antimicrobial peptides for fighting against microbe-associated metalloproteinases during the humoral immune response. This species has been explored as an insect model system for in vivo testing for human pathogens causing lethal diseases like *Listeria* [105, 106], as a system for the discovery of anti-infective drugs [107] and for discovery of natural antibiotics. Such an insect model system would be more cost-effective than a mammalian testing system; further, it would be more ethically acceptable, providing comparable data that would be of great benefit for medical diagnostics and therapeutics.

6.5 Conclusions

The emergence of multiresistant bacterial strains has demonstrated the need for alternatives to synthetic antibiotics, and this has led to the discovery of natural antimicrobial peptides from the order Lepidoptera to fight against microorganisms. Although with limited success in clinical trials, knowledge acquired in the past two decades has led to the identification of close to 1750 natural AMPs, among which a significant number are from insects and were first isolated from the order Lepidoptera. The AMPs presented in this chapter will provide further insights for designing anti-infective drugs against cancer, wound healing, anti-inflammatory disorders, antibacterial drugs, and food preservatives; moreover, much higher efficacy can be achieved through a synergistic approach. Interestingly, to date, clinical trials have already shown limited resistance against these peptides among pathogenic bacterial strains, greater ease of synthesis, and discovery of novel mechanisms of action. Such significant properties of AMPs make them a powerful arsenal of molecules that could be transformed into novel antimicrobial agents for the twenty-first century.

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Chapter 7
Advanced Protein Expression Using *Bombyx mori* Nucleopolyhedrovirus (BmNPV) Bacmid in Silkworm

Tatsuya Kato and Enoch Y. Park

Abstract An expression system using a *Bombyx mori* nucleopolyhedrovirus (BmNPV) bacmid has been developed and used for efficient expression of protein using silkworm. Silkworm can sustain large-scale production of recombinant proteins due to its ease of rearing and scaling-up. Our chapter focuses on the modification of a BmNPV bacmid for a more efficient protein expression system. For example, we discuss how to achieve construction of a stronger promoter, less proteolytic degradation of expressed proteins, and a chaperone-coexpressed expression system. We describe the application of functional BmNPV particles purified from silkworm hemolymph to vaccines, antibody production, and transmembrane protein analysis. For human use, the major problem of proteins produced in silkworm is contamination by adventitious agents and protein quality. Of special concern is that *N*-glycosylation in silkworms is of a high-mannose type in most cases, which is different from the complex type found in mammals. We end by looking to future prospects for integration/applications of protein expression systems with silkworm biotechnology.

Abbreviations

α4GnT	Human α1,4-*N*-acetylglucosaminyltransferase
AcMNPV	*Autographa californica* multiple nucleopolyhedrovirus
ADCC	Antibody-dependent cell-mediated cytotoxicity
β3GnT	Human β1,3-*N*-acetylglucosaminyltransferase
BiP	Human heavy chain-binding protein
Bm	*Bombyx mori*
BmNPV	*Bombyx mori* nucleopolyhedrovirus
BmNPV CP^- (CP^-)	Cysteine protease-deficient BmNPV

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BmNPV CP^- Chi^- (CP^-Chi^-)	Cysteine protease- and chitinase-deficient BmNPV
CNX	Human calnexin
CRT	Human calreticulin
CSFV	Classical swine fever virus
DDS	Drug delivery system
ELISA	Enzyme-linked immunosorbent assay
ERp	Human ERp57
FMDV	Foot-and-mouth disease virus
GFP	Green fluorescent protein
HA	Hemagglutinin
Hsp70	Heat shock protein 70
IgG	Immunoglobulin G
IL-5	Interleukin-5
LUV	Large unilamellar vesicle
MSG	Middle silk gland
PIT, 7d	Post-injection time, 7 days
PSG	Posterior silk gland
RSV	Rous sarcoma virus
RV	Rabies virus
SAG1	Surface antigen 1
SRB	Sulforhodamine B
SRS2	Surface antigen 1-related sequence 2
ST6Gal1	α2,6-sialyltransferase 1
VLP	Virus-like particle

7.1 Introduction

Insects and insect cells have been widely used for recombinant protein production. Mostly, baculoviruses, which have only species-specific tropisms, have been also used in these systems. Among these baculoviruses, *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) is the first choice to express recombinant proteins in conventional insect cells such as Sf-9 cells (from *Spodoptera frugiperda*) and High Five cells (from *Trichoplusia ni*). In order to produce recombinant proteins, the gene of interest is inserted into the genome of AcMNPV. At the beginning of this research, the gene of interest and AcMNPV genomic DNA were co-transfected into Sf-9 cells to generate recombinant AcMNPVs. Nowadays, the AcMNPV bacmid was developed as a Bac-to-Bac system, supplied by Life Technologies. This bacmid is a baculovirus shuttle vector, which can be propagated in *Escherichia coli*, and the recombinant bacmid can be generated in *E. coli*. Recombinant AcMNPV can also be generated by transfection into Sf-9 cells for several days. Using this bacmid system, rapid recombinant protein production can be achieved, compared to the conventional method.

Silkworms, notably the species, *Bombyx mori*, have also been used for recombinant protein expression using another baculovirus, *Bombyx mori* nucleopolyhedrovirus (BmNPV). Silkworms, which have been domesticated by humans for thousands of years for silk production, need human care to survive and can no longer survive on their own. For a long time, silkworms were used as economically important insects throughout the world, especially in East Asia. Recently, "silkworm biotechnology" has been developed to produce recombinant proteins in silkworms. Silkworms have a high capacity to produce recombinant proteins as bioreactors and their capacity exceeds that of other expression systems. In addition, large-scale production of recombinant proteins using silkworms can be achieved easily because of the convenience of rearing, in which only the number of silkworms needs to be increased. In general, to produce recombinant proteins in silkworms, BmNPV is used [1, 2]. Recombinant BmNPV harboring a foreign gene is constructed through transfection by introducing the BmNPV genome together with a gene transfer vector into cultured Bm cells. This method hampers extensive use of silkworms to produce recombinant proteins because the construction of recombinant BmNPV is cumbersome and takes a long time (up to half a year). However, in 2005 Motohashi and coworkers developed a BmNPV Bac-to-Bac system (BmNPV bacmid) similar to the AcMNPV system which makes recombinant protein production in silkworms readily accessible [3]. Using this BmNPV bacmid system, it takes only two weeks from gene amplification by PCR to the production of recombinant protein, which is comparable to a system using cultured insect cells. The silkworm expression system makes rapid and large-scale recombinant protein expression compatible. Moreover, the establishment of transgenic silkworms to produce recombinant proteins has been achieved. In this chapter we introduce the development and improvement of silkworm biotechnology used to produce recombinant proteins and its application to various fields. In addition, we briefly introduce the production of functional BmNPV particles in silkworm larvae.

7.2 Protein Expression in Silkworms

7.2.1 Using BmNPV or BmNPV Bacmid

Over 10 years ago, BmNPV was routinely used for expression of recombinant proteins in silkworms [1, 2]. In these cases, the protein of interest was expressed using its own signal peptide to secrete it into hemolymph. For example, human parathyroid hormone was expressed into hemolymph in silkworm larvae in an active form using native pre- and pro-domains [4]. These results showed that silkworms have a similar processing system to mammalian cells. Sometimes other heterologous signal peptides (β-interferon signal peptide, yeast α-factor prepro-domain, AcMNPV GP64 glycoprotein signal peptide, and so on) were successfully adopted for the secretory expression of recombinant proteins in silkworm larvae [5–7]. However,

some signal peptides of secretory proteins or peptides from insects do not work in silkworms to secrete expressed proteins even if they work as expected in Sf-9 and High Five cells. For example, Park et al. [8] showed that the signal peptides of melittin from honeybee, GP64 glycoprotein from AcMNPV, and cecropin from *Hyalophora cecropia* did not allow a GFP fusion protein to be secreted into cultured supernatant in Bm5 cells, but the signal peptides of bombyxin and prophenoloxidase-activating enzyme from *B. mori* did. This indicates that indigenous signal peptides should be favorable for expressing secretory recombinant proteins in silkworm larvae as well.

In hemolymph, expressed recombinant proteins are often prone to attack and degradation by proteases. BmNPV has its own cysteine protease to liquefy silkworm tissues in the very late stage of infection. To minimize the degradation of expressed proteins, BmNPV deficient in cysteine protease was constructed [9]. Using this cysteine protease-deficient BmNPV, rat IL-5 was expressed in silkworm hemolymph, overcoming the lack of expression of rat IL5 using wild-type BmNPV [10]. A cysteine protease-deficient BmNPV (BmNPV CP^{-}) bacmid was also developed in which the degradation of GFP fusion protein in hemolymph was partially but not completely suppressed [11]. Moreover, a cysteine protease- and chitinase-deficient BmNPV (BmNPV $CP^{-}Chi^{-}$) bacmid was also developed to improve the yield of recombinant proteins expressed in silkworms [12]. The removal of chitinase and cysteine protease activities significantly delays the liquefaction of silkworm larvae in the late stages of BmNPV infection and improves the yield of expressed recombinant proteins (Fig. 7.1). Chitinase and protease activities of hemolymph of silkworm larvae injected with BmNPV $CP^{-}Chi^{-}$ bacmid were also suppressed compared to those associated with the wild-type BmNPV bacmid.

In baculovirus expression systems, typically a polyhedrin promoter is used, and sometimes a p10 promoter, which works at a very late stage of baculovirus production, similar to the polyhedrin promoter, is also adopted. To enhance the level of recombinant protein expression, a modified polyhedrin promoter (Fig. 7.2a) was developed. A burst sequence contains the sequence between the transcriptional initiation site around TAAG and the translational initiation sequence specific to the untranslated leader sequence of very late p10 promoter. Manohar et al. [13] showed that a polyhedrin promoter modified by adding 9 AT-rich burst sequences needed for activation of late expression enhanced the expression of GFP_{uv}-human β1,3-*N*-acetylglucosaminyltransferase 2 fusion protein in silkworm larvae (Fig. 7.2b), compared to using a normal polyhedrin promoter. The expression of human α2,6-sialyltransferase was improved slightly using a modified polyhedrin promoter having 5 burst sequences (Figs. 7.1 and 7.2C), but suppressed using a modified polyhedrin promoter having 9 burst sequences [14]. These results suggest that the improvement of recombinant protein expression in silkworm using modified promoters depends on the properties of the recombinant protein itself. These modified promoters can also be used for recombinant protein expression in cultured insect cells using the recombinant AcMNPV bacmid system. In another report, a VP39 promoter modified with a homologous DNA region, HR3, ameliorated the aggregation of human sphingosine kinase and 2-oxoisovalerate dehydrogenase α-subunit

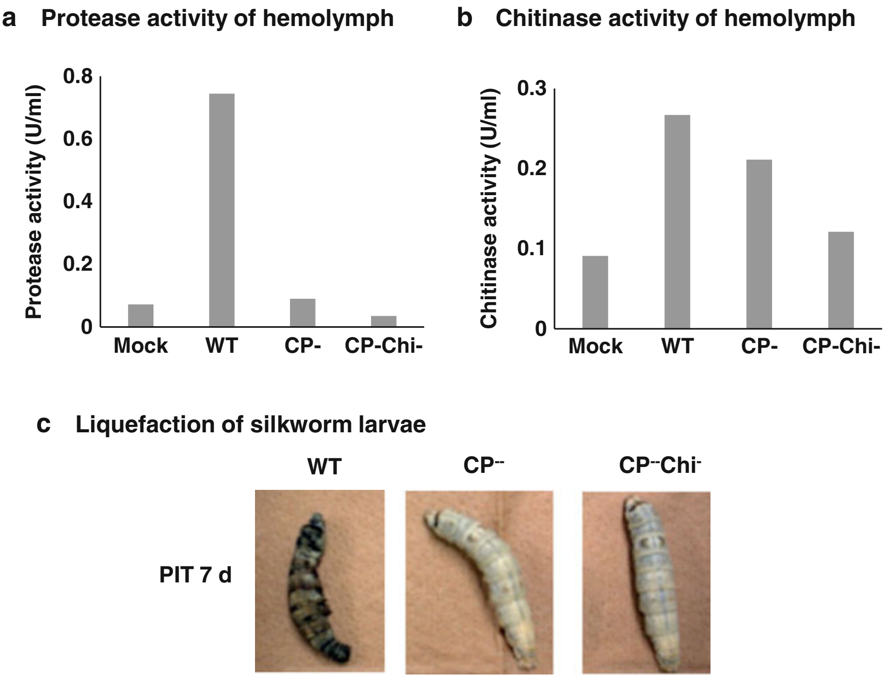


Fig. 7.1 Properties of BmNPV bacmid, cysteine protease-deficient BmNPV bacmid, and cysteine- and chitinase-deficient BmNPV bacmid. (**a**) Protease activity of hemolymph from silkworm larvae injected with BmNPV bacmid DNA. (**b**) Chitinase activity of hemolymph from silkworm larvae injected with BmNPV bacmid DNA. (**c**) Liquefaction of silkworm larvae injected with BmNPV bacmid DNA. Mock, silkworm larvae without injection of any BmNPV bacmid; CP^-, cysteine protease-deficient BmNPV; CP^-Chi^-, cysteine protease- and chitinase-deficient BmNPV; PIT, post-injection time; 7d, 7 days

expressed in BmN cells and mouse IgG1 in silkworm larvae [15]. The choice of promoters is also important to obtain soluble and active recombinant proteins in BmN cells and silkworms.

A chaperone co-expression strategy has been adopted in various expression systems and can also be utilized to improve the production and yield of recombinant proteins in silkworm biotechnology. The expression level of GFP-fused human α1,4-*N*-acetylglucosaminyltransferase was increased by 1.4- and 2.0-fold with the co-expression of human calnexin and human immunoglobulin heavy chain-binding protein, respectively, under the control of a polyhedrin promoter (Fig. 7.3a) [16]. In addition to these two chaperones, human calreticulin enhanced the expression level of human IgG in silkworm larvae even if human calreticulin was expressed under the very early IE2 promoter of *Orgyia pseudotsugata* multicapsid nucleopolyhedrosis virus (Fig. 7.3b) [17, 18]. These human chaperones also work in cultured insect cells to enhance the expression level of recombinant proteins [19–22] and in insect cells and insects for the improvement of recombinant protein production. In our

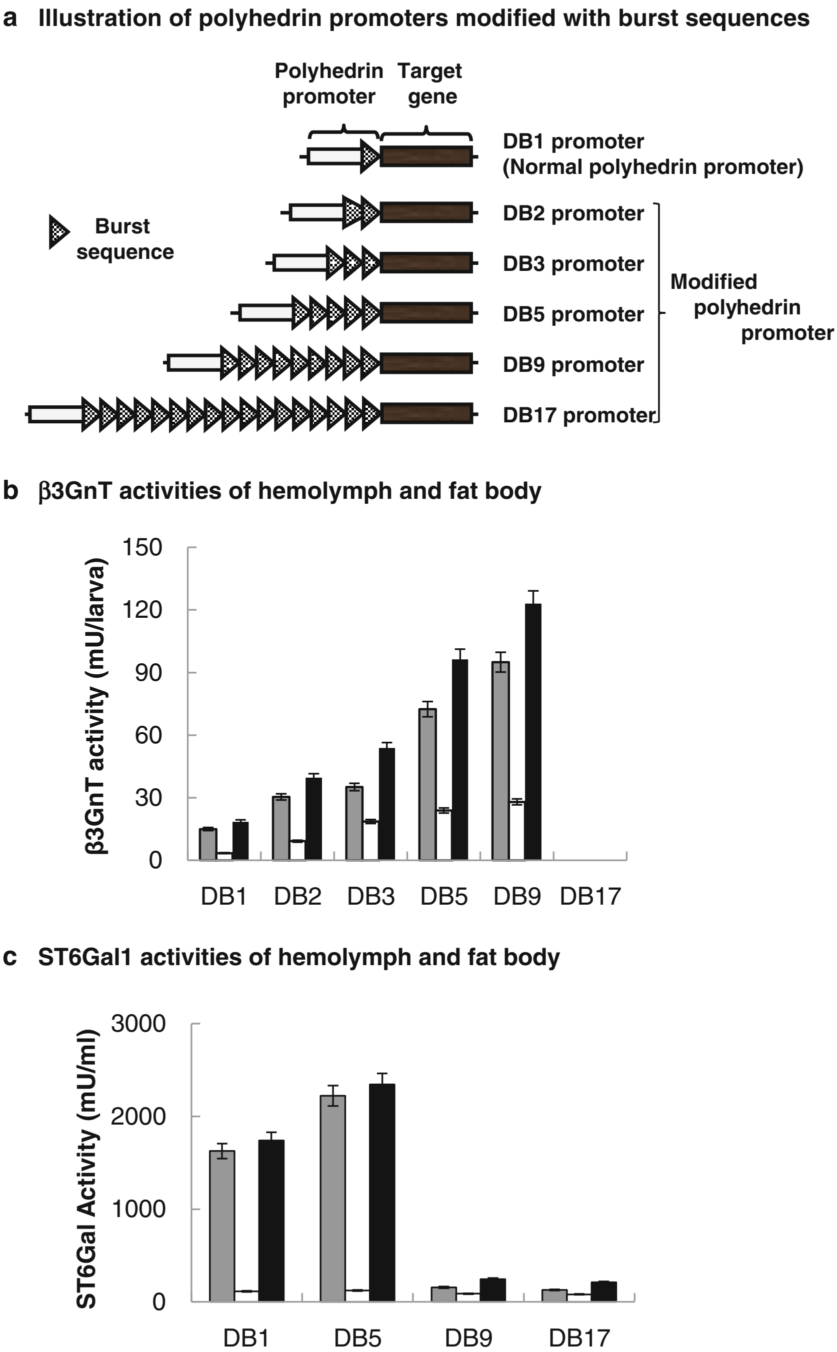


Fig. 7.2 Protein expression in silkworm larvae using burst sequence-modified polyhedrin promoters. (**a**) Diagrams of polyhedrin promoters modified with burst sequences. (**b**) β3GnT activity of hemolymph (*gray bar*) and fat body (*white bar*) from silkworm larvae injected with each BmNPV. *Black bars* indicate the summation of both activities in hemolymph and fat bodies [13]. (**c**) ST6Gal1 activity of hemolymph (*gray bar*) and fat body (*white bar*) from silkworm larvae injected with each BmNPV. *Black bars* indicate the summation of both activities in hemolymph and fat bodies [14]. β3GnT, human β1,3-*N*-acetylglucosaminyltransferase; ST6Gal1, rat α2,6-sialyltransferase 1

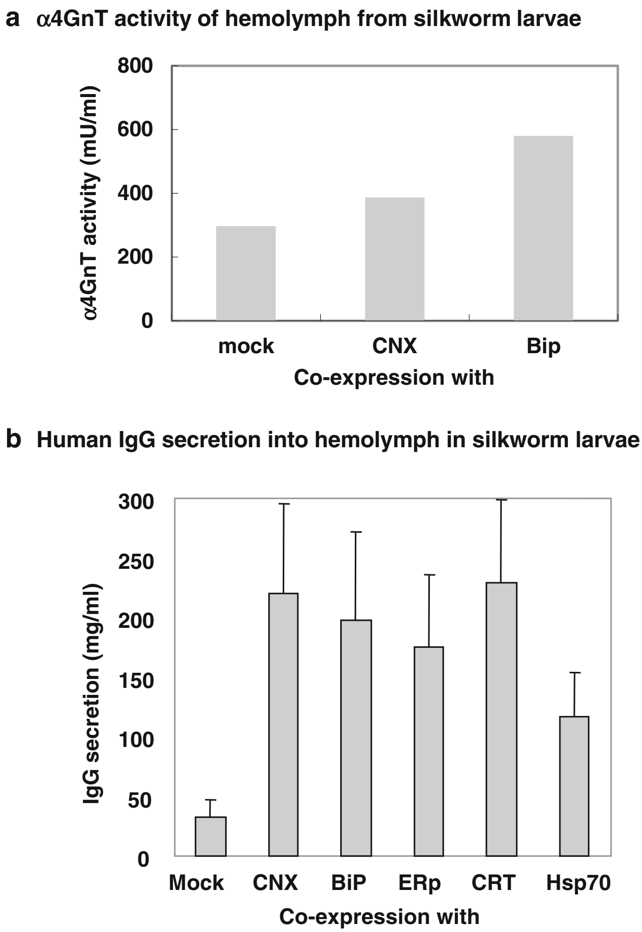


Fig. 7.3 Effect of chaperone co-expression on recombinant proteins in silkworm larvae. (**a**) α4GnT activity of hemolymph from silkworm larvae when human α4GnT and various chaperones were co-expressed [16]. Each chaperone was expressed under the control of a polyhedrin promoter. (**b**) Secretion of human IgG into silkworm larval hemolymph with the co-expression of various chaperones [17]. Each chaperone was expressed under the control of the very early IE2 promoter of the *Orgyia pseudotsugata* multiple nucleopolyhedrovirus. CNX, human calnexin; BiP, human heavy chain-binding protein; ERp, human ERp57; CRT, human calreticulin; Hsp70, human heat shock protein 70; α4GnT, human α1,4-*N*-acetylglucosaminyltransferase; IgG, Immunoglobulin G

recent research, improvement of the quality of recombinant protein bearing disulfide bonds was observed in silkworm larvae by the co-expression of human protein disulfide isomerase (unpublished data). Co-expression of chaperones is a crucial way to express sophisticated foreign proteins which require some co- and posttranslational modification.

In many cases, secretory proteins, including soluble transmembrane proteins lacking their own transmembrane domains, have been expressed in silkworm larvae and purified from larval hemolymph because of the ease of obtaining them as a

"protein solution" [23–25]. Even transmembrane proteins have been expressed in silkworm larval hemolymph [26, 27]. Additionally, BmNPV can amplify not only in hemocytes but also in fat body of silkworm larvae. Therefore, most recombinant transmembrane proteins are expressed and localized in the fat body, from which human (pro)renin receptor was purified [26].

BmNPV also infects silkworm pupae, and some recombinant proteins have been expressed in this silkworm stage [28–31]. A pupa is an alternative way to produce recombinant proteins conveniently because the protein yield is comparable to that of silkworm larvae. Pupae can be stored at 4 °C for a month to produce recombinant proteins. Moreover, unlike silkworm larvae, no diet is needed for pupal incubation. However, comparing these two stages, *N*-glycans of recombinant glycoprotein purified from silkworm pupae are a little different from those from silkworm larvae [17]. Human IgG purified from silkworm pupae had a 3.5-fold higher level of β1,2-linked GlcNAc than that obtained from silkworm larvae. This indicates that silkworm pupae are better for expressing mammalian glycoproteins in terms of the *N*-glycan structures than silkworm larvae. However, *N*-glycan structures are dependent on the properties of each glycoprotein; for example, human β1,3-*N*-acetylglucosaminyltransferase 2 purified from the hemolymph of silkworm larvae had several complex types of *N*-glycans, but human α2,6-sialyltransferase purified from the hemolymph of silkworm larvae had only paucimannose types of *N*-glycans [32, 33].

The expression of a protein complex is still a challenging problem because several subunits must be expressed simultaneously in a cell, and a sufficient quality and quantity of purified recombinant protein complex is often vitiated by multi-protein expression. To overcome this problem, simultaneous multi-protein expression systems using silkworms have been developed. The MultiBac expression system [34] is tailored to express multi-protein complexes in a baculovirus expression system. Several gene expression cassettes can be inserted into one AcMNPV bacmid using this MultiBac expression system through Cre-loxP recombination. Virus-like particles composed of three structural proteins from rotavirus and human DNA polymerase delta were expressed in silkworm larvae using a BmNPV bacmid system improved by use of the MultiBac system [35, 36]. Human microsomal transfer protein-human PDI complex was expressed in the fat body of silkworm larvae using constructed gcLINK vectors [29]. gcLINK vectors containing gcLINK sequences can combine several gene expression cassettes via ligation-independent cloning which facilitates the expression of multi-protein complexes in silkworms. These techniques accelerate the detailed analysis of eukaryotic protein complexes, which cannot be purified from native sources and expressed recombinantly in high enough amount to analyze in detail.

7.2.2 Transgenic Silkworms

Recently, recombinant protein production has also been carried out using transgenic silkworms. Tamura et al. developed the methodology for silkworm transgenesis using a *PiggyBac* transposon-derived vector [37, 38]. In this system, the gene of

interest is inserted between inverted sequences of *PiggyBac* in a constructed vector which is injected into eggs with a helper plasmid harboring the *PiggyBac* transposase gene. Using this system, several recombinant proteins have been produced in silkworm cocoons [39]. Typically 1–150 μg of recombinant protein/mg cocoon weight are obtained using this expression system. For recombinant protein production in cocoons, recombinant proteins should be expressed in silk glands. For protein expression in the posterior silk gland (PSG), recombinant proteins are fused with one of the 3 predominant silk fiber proteins normally produced there, fibroin H-chain or L-chain or fibrohexamerin, and are expressed in the inner fibroin core using a fibroin H-chain or L-chain or fibrohexamerin promoter [40–43]. On the other hand, recombinant proteins will localize in the outer sericin layer when they are expressed in the middle silk gland (MSG) under the control of a sericin 1 promoter [44]. No fusion partner is needed to secrete recombinant proteins expressed in the MSG into the cocoon. Moreover, these recombinant proteins can be extracted directly from cocoons by soaking in phosphate-buffered saline or Tris-buffered saline [44, 45].

Mouse monoclonal antibody can be also expressed as an H_2L_2 form in cocoons (MSG), extracted with 3 M urea and purified as an active form by protein G affinity chromatography [46]. *N*-glycans of mouse monoclonal antibody purified from cocoons are not fucosylated as in the native form. Fucose residues in the *N*-glycans of antibodies compromise antibody-dependent cell-mediated cytotoxicity (ADCC) activity, which is one of the mechanisms of cell-mediated immune defense. This indicates that α-1,3- and α-1,6-fucosyltranferase are not expressed in MSG, in contrast to recombinant proteins expressed in hemolymph and fat body, which are normally fucosylated. In addition, terminally di-*N*-acetylglucosaminated glycan was observed in contrast to *N*-glycans of recombinant glycoproteins purified from silkworm fat body and hemolymph, which are paucimannose and hybrid types. This also indicates that *N*-acetylglucosaminyltransferases I and II are expressed in MSG. Precise glycosylation is important for production of mammalian monoclonal antibody because α1,3-linked fucose induces high antigenicity in humans, and the removal of α1,6-linked fucose from IgG enhances its antibody-dependent cellular cytotoxicity activity. Consequently, the MSG expression system in transgenic silkworms is better for producing therapeutic monoclonal antibodies than fat body or hemolymph.

7.3 Application of Recombinant Protein Produced in Silkworms

Silkworm larvae and pupae can produce recombinant proteins, BmNPV particles and virus-like particles for use as products in various fields (Fig. 7.4). Some examples of these applications are described below.

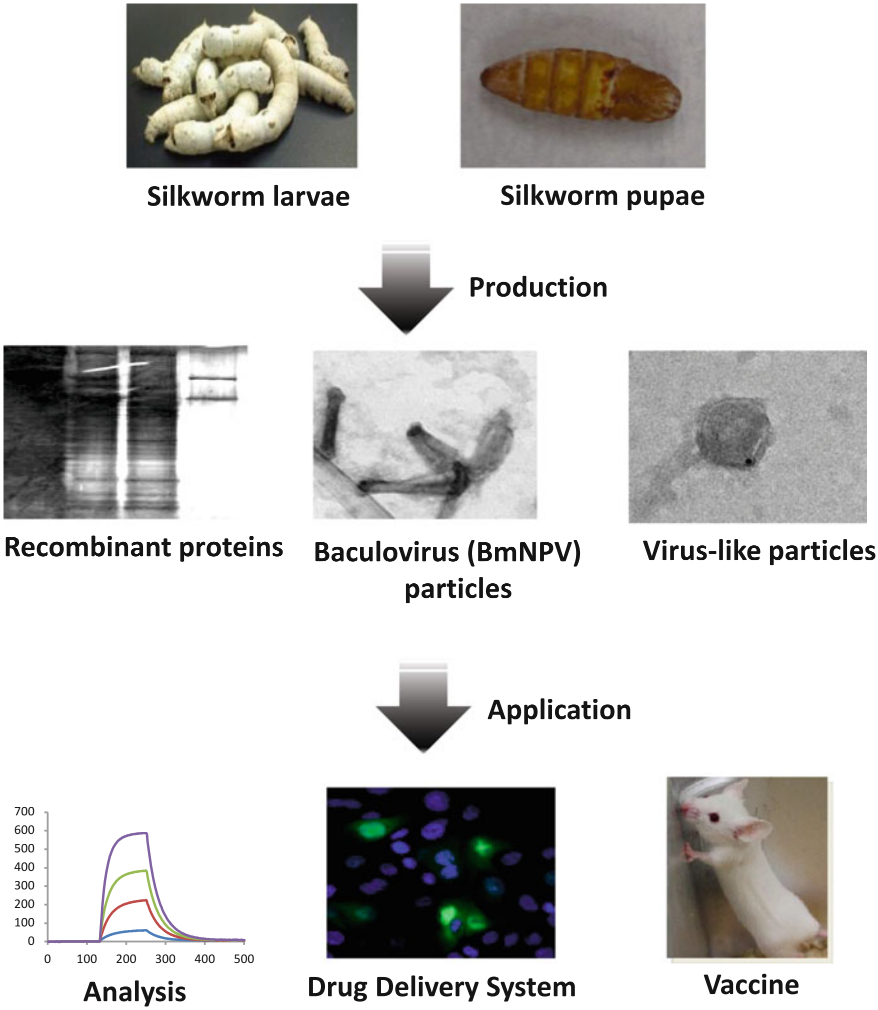


Fig. 7.4 Application of products from silkworm larvae and pupae

7.3.1 Recombinant Proteins for Therapeutics

Many recombinant proteins have been produced in silkworm, as described above, with applications in various fields, chiefly for diagnostic uses in human and animal medicine. Equine influenza virus hemagglutinin was purified from silkworm larvae using fetuin-affinity chromatography and utilized to detect hemagglutinin (HA)-specific antibody in horse sera using enzyme-linked immunosorbent assay (ELISA) for serological diagnosis [47]. Bovine tissue factor expressed in silkworm pupae was also characterized for its diagnostic use; the properties of Owren-type

prothrombin time reagent incorporated by this recombinant bovine tissue factor were similar to commercial products [48]. In this case, lot-to-lot variability of the purified recombinant protein (3 lots) was very small, indicating it is possible that recombinant silkworm proteins can be used for reliable large-scale production for diagnostic use. For future therapeutic applications, silkworm larvae were also used to produce large amounts of human butyrylcholinesterase [49]. However, few products produced in silkworms have been commercialized. An exception is feline interferon from silkworm larvae, which was first commercialized in 1994 as a veterinary medicine, and subsequently, canine ϒ-interferon was commercialized in 2005 [50]. These interferons have already been approved by the European Medicines Agency, Ministry of Agriculture, Forestry and Fisheries of Japan, and other agencies in 20 countries. This suggests that silkworms can generate commercial therapeutics for human in the future. However, it is difficult to purify intact recombinant protein from silkworms because it is prone to degradation [51]. To make the most of producing a high level of recombinant proteins in silkworms, the recombinant protein and purification systems must be improved. Additionally, silkworm strains with higher yields for recombinant protein expression have to be developed [12, 52].

7.3.2 Vaccines

The vaccine technique has been developed to help the immune system defend against infectious viruses and microorganisms. Traditionally, live-attenuated and inactivated pathogens have been used as vaccines. Although highly efficacious to treat infectious pathogens, such vaccines have safety and cost problems and take a long time to prepare. To circumvent these problems, subunit vaccines have been developed and produced in various expression systems. In silkworms, proteins from infectious viruses and animal parasites have been produced as subunit vaccines using a BmNPV baculovirus expression system and BmNPV bacmid system. Foot-and-mouth disease virus (FMDV) polypeptide (P1-2A domain and 3C protease) was expressed in silkworm larvae using a BmNPV baculovirus expression system. Hemolymph expressing FMDV polypeptide which was directly used for cattle vaccination [53] induced the production of FMDV-specific antibody and protected cattle from viral infection. In addition, virus-like particles of FMDV from silkworm larvae and pupae infected with recombinant BmNPV were also used successfully for cattle vaccination [54]. Nucleoprotein of rabies virus (RV) produced by a BmNPV baculovirus expression system and purified from silkworm pupae using Affi-Gel 10 and gel filtration chromatography allowed mice to produce RV-specific antibody and protect mice from RV challenge [55]. Immunization of surface antigen 1 (SAG1) and SAG1-related sequence 2 (SRS2) of *Neospora caninum* produced by BmNPV bacmid system and purified from silkworm hemolymph using affinity chromatography induced SAG1- and SRS2-specific antibody in mice (Fig. 7.5) [56]. Thus each *N. caninum* antigen purified from silkworm hemolymph also mitigated the infection of *N. caninum* in mice [57]. Envelope glycoprotein E2 of

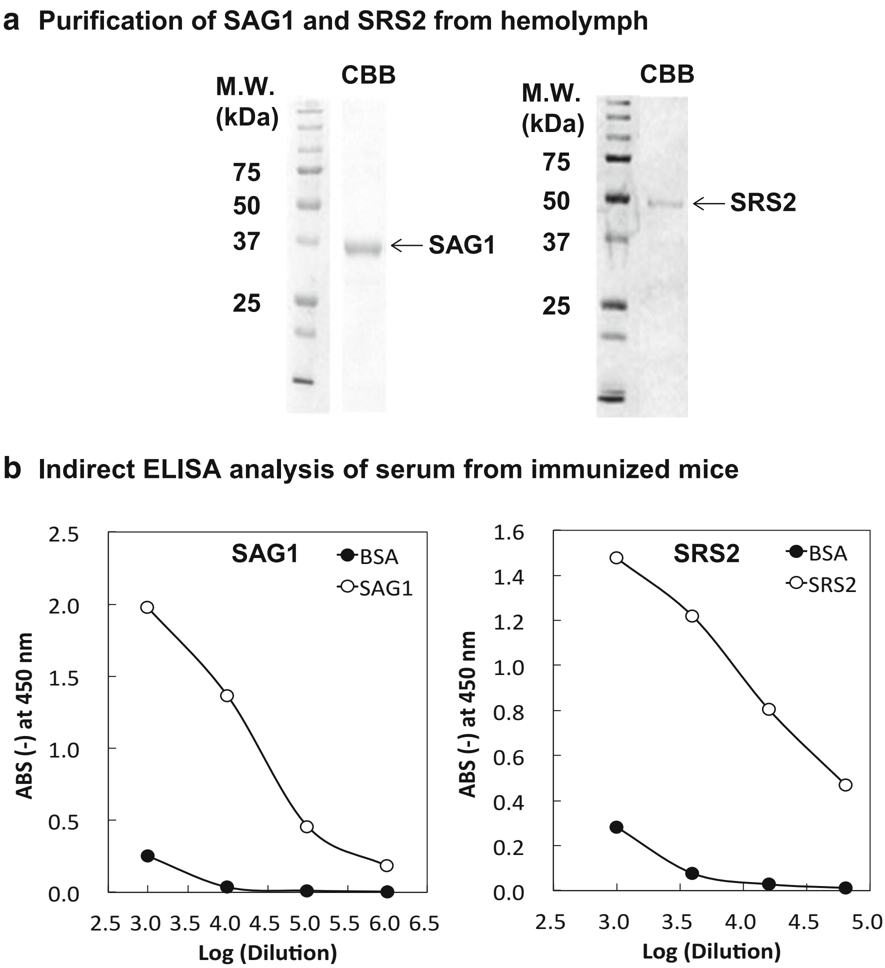


Fig. 7.5 Application of protozoal antigens produced in silkworm larvae as a vaccine. (**a**) Purification of FLAG-tagged NcSAG1 and NcSRS2 from hemolymph using anti-FLAG M2 antibody agarose [56]. (**b**) Indirect ELISA analysis of serum from mice immunized with purified NcSAG1 or NcSRS2 [56]. NcSAG1, surface antigen 1 from *N. caninum*; NcSRS2, SAG1-related sequence 1 from *Neospora caninum*; CBB, Coomassie Brilliant Blue stain; BSA, bovine serum albumin

classical swine fever virus (CSFV), which was expressed in silkworm larvae as a fusion protein with polyhedrin and purified by His-Trap column after alkaline solubilization and cleavage by Factor X, induced the production of E2-specific antibody in mice [58]. Oral administration of freeze-dried powder of pupae expressing VP6 protein from grass carp reovirus produced a specific antibody to VP6 protein [59]. In most cases, recombinant antigens produced in silkworm larvae and pupae have been used highly successfully for animal vaccination to protect from virus or

parasite infection in animals even if they are not purified from silkworm larvae and pupae. The lack of need to purify recombinant antigens leads to antigen production in silkworms with lower cost.

7.3.3 Virus-like Particles

Virus-like particles (VLPs) are composed of the structural proteins of viruses and have almost the same shape as native viruses without any genetic material of native viruses. Currently VLPs are the most promising candidates for vaccines against pathogenic viruses and parasites because of their self-adjuvanting properties and functional versatility [60]. VLPs have been produced in various expression systems including silkworms. As described above, VLPs of FMDV were produced in silkworm larvae and pupae; particles were formed at a diameter of 30 nm and immunized as a vaccine to FMDV in cattle to protect from infection [49, 54]. The VP2 protein of canine parvovirus expressed in silkworm pupae was self-assembled into VLPs with a diameter of 25–30 nm even if the virus-neutralizing epitope of rabies virus was inserted into the loop domain of VP2 protein [61]. Three structural proteins (VP2, VP6, and VP7) of rotavirus were simultaneously expressed in silkworm larvae using a baculovirus-silkworm multigene expression system, and formed rotavirus VLPs were purified by CsCl gradient centrifugation with a yield of 12.7 μg per larva [35]. FMDV, canine parvovirus, and rotavirus belong to non-enveloped virus. VLPs are self-assembled and form when each viral capsid protein is expressed in silkworms. In the case of an enveloped virus, *Rous sarcoma* virus (RSV) gag VLPs displaying SRS2 of *N. caninum* on their surface through a glycosylphosphatidylinositol anchor were produced in the hemolymph of silkworm larvae (Fig. 7.6), and serum from mice immunized with these VLPs without any adjuvant was used to detect *N. caninum*-specific antibody in bovine serum, suggesting that these VLPs can be utilized as a vaccine candidate for neosporosis in cattle [27]. It was also reported that an RSV gag VLP displaying a colon carcinoma cell-specific single-chain antibody was used as a carrier for a drug delivery system (DDS) [62] for sulforhodamine B (SRB), a fluorescent cell imaging agent. These VLPs were associated with large unilamellar vesicles (LUVs) to enable them to carry SRB, and the VLPs-LUV complex containing SRB was specifically carried to LS174T cells (colon carcinoma cells). This suggests that VLPs purified from silkworms can be used as a drug carrier for DDS. These RSV gag VLPs were also used for a binding assay for the human transmembrane protein, (pro)renin receptor [63].

7.3.4 BmNPV Particles

Baculovirus particles have been utilized as functional nanoparticles, gene and drug delivery vesicles, and vaccines [64, 65]. Baculoviruses are not pathogenic and do not replicate in mammalian cells. However, these viruses can convey genetic

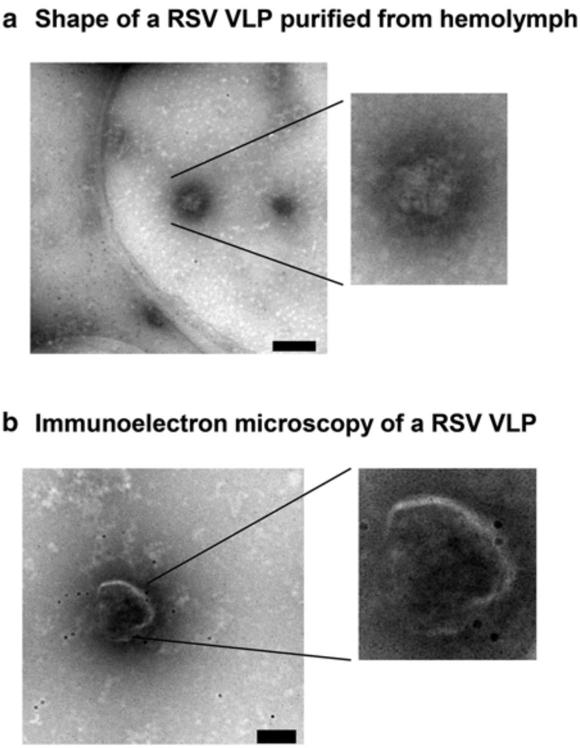


Fig. 7.6 Virus-like particles produced in silkworm larvae. (**a**) Purified RSV gag VLP displaying NcSRS2 observed under transmission electron microscopy with negative staining [27]. Scale bar is 200 nm. (**b**) Immunoelectron microscopy of RSV gag VLP displaying NcSRS2 using mouse anti-*Neospora* antibodies with negative staining [27]. Scale bar is 100 nm. RSV, Rous sarcoma virus; VLP, viruslike protein; NcSRS2, SAG1-related sequence 1 from *Neospora caninum*

material and produce recombinant proteins in mammalian cells using mammalian-derived promoters. Moreover, humans do not have preexisting antibodies to baculoviruses, indicating that baculoviruses can escape preexisting immunity in humans. To use baculovirus particles for delivery vesicles and vaccines, a large-scale production system is indispensable to obtain sufficient quantities of particles. Silkworm larvae have the potential for large-scale production of not only recombinant proteins but also functional BmNPV particles (Fig. 7.7). Silkworm larvae can produce a much higher titer (10^8–10^9 pfu/ml) of BmNPV particles in hemolymph than cultured cells, and BmNPV particles can be purified from hemolymph using Sephacryl S-1000 size exclusion chromatography [66]. Although this system is very efficient to prepare large amounts of baculovirus particles, it has to be evaluated in more detail for particle quality and impurity. As another application, human (pro)renin receptor-displaying BmNPV particles purified from hemolymph were applied to a human renin-binding assay [67]. In another application, mice were immunized with BmNPV particles displaying an *N. caninum* antigen partially purified from silkworm larval hemolymph followed by infection with the parasite [68]. Exposure to the antigen-displaying BmNPV particles reduced the cerebral infection of *N. caninum* significantly compared to controls using Freund's incomplete adjuvant [57]. These examples illustrate some of the many potential applications for use of BmNPV particles expressing recombinant proteins or peptides in medical and related areas.

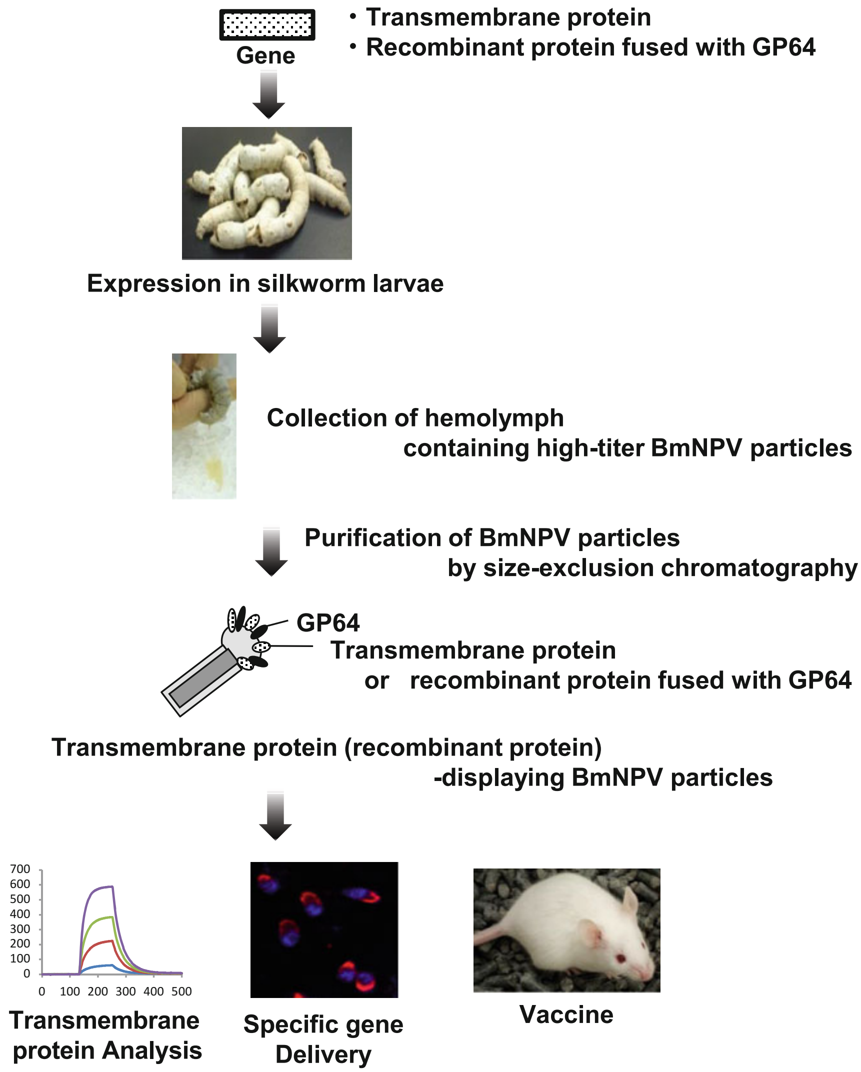


Fig. 7.7 Preparation of recombinant protein-displaying BmNPV particles

7.4 Conclusions and Future Prospects

The baculovirus expression system has been used widely for recombinant protein production. Silkworms are especially well adapted for large-scale production of recombinant proteins because of their ease of rearing and scaling-up. Recently, transgenic silkworm systems have been developed to produce recombinant proteins in cocoons, which make the extraction and purification of recombinant protein very

easy. In addition, functional BmNPV particles purified from silkworm hemolymph can be used for drug and gene delivery, vaccines, and antibody production. Although the protein production capacity of silkworms exceeds that of other systems, for human use the major problem is contamination by adventitious agents and protein quality. Especially problematic is that *N*-glycosylation in silkworms is a paucimannose type in most cases, which is different from the complex type found in mammals. In addition, recombinant proteins are prone to undergo degradation in silkworms. The addition of *N*-glycans to recombinant proteins and protein degradation often cause their heterogeneity and deteriorate their quality. These problems must be overcome for ultimate human use. However, silkworms lead in the large-scale production of recombinant proteins with low cost and high yield as potential medicines for human therapeutics in the near future.

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Chapter 8
Insect Biotechnology

Anthony O. Ejiofor

Abstract For the purpose of this work, insect biotechnology, which is also known as yellow biotechnology, is the use of insects as well as insect-derived cells or molecules in medical (red biotechnology), agricultural (green biotechnology), and industrial (white) biotechnology. It is based on the application of biotechnological techniques on insects or their cells to develop products or services for human use. Such products are then applied in agriculture, medicine, and industrial biotechnology. Insect biotechnology has proven to be a useful resource in diverse industries, especially for the production of industrial enzymes including chitinases and cellulases, pharmaceuticals, microbial insecticides, insect genes, and many other substances. Insect cells (ICs), and particularly lepidopteran cells, constitute a competitive strategy to mammalian cells for the manufacturing of biotechnology products. Among the wide range of methods and expression hosts available for the production of biotech products, ICs are ideal for the production of complex proteins requiring extensive posttranslational modification. The progress so far made in insect biotechnology essentially derives from scientific breakthroughs in molecular biology, especially with the advances in techniques that allow genetic manipulation of organisms and cells. Insect biotechnology has grown tremendously in the last 30 years.

Abbreviations

AAV	Adeno-associated virus
AcMNPV	*Autographa californica* multinuclear polyhedrosis virus
Bt	*Bacillus thuringiensis*
BV	Baculovirus
Cry	Crystal protein
EGFP	Enhanced green fluorescent protein
FXR	Farnesoid X-activated receptor
GFP	Green fluorescent protein

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GM	Genetically modified
HR	Herbicide resistant
BEV	Baculovirus expression vector
IC-BEVS	Insect cell baculovirus expression vector system
IC	Insect cell
IR	Insect resistant
LXR	Liver X receptor
LV	Lentivirus
MOI	Multiplicity of infection
MRSA	Methicillin-resistant *Staphylococcus aureus*
NPV	Nucleopolyhedrosis virus
rAAV	Recombinant adeno-associated virus
rTRAIL	Recombinant TNF-related apoptosis-inducing ligand
rBV	Recombinant baculovirus
VEGF	Vascular endothelial growth factor
VLP	Viruslike particle

8.1 Introduction and Scope

For the purpose of this work, insect biotechnology, which is also known as yellow biotechnology [1–3] in contrast to green (plant), red (animal), and white (industrial) biotechnology, is discussed as the application of biotechnological techniques on insects or their cells to develop products or services for human use. Such products are then applied in agriculture, medicine, and industrial biotechnology [3–6]. Insect biotechnology has proven to be a useful resource in diverse industries, especially for the production of industrial enzymes, microbial insecticides, insect genes, and many other substances. Insect cells (ICs), and particularly lepidopteran cells, constitute a competitive strategy to mammalian cells for the manufacturing of biotechnology products. Among the wide range of methods and expression hosts available for the production of biotech products, ICs are ideal for the production of complex proteins requiring extensive posttranslational modification.

The development of the field was driven by the importance of insects in all fields of human endeavor. Insects are the most diverse of all groups of organisms, with more species than any other. The number of extant species is estimated at about 1.9×10^6 and potentially represents over 90 % of the various animal life forms on Earth [7]. They have overwhelming impact on agriculture and public health. Insect biotechnology uses biotechnological methods on insects (or their cells) to develop products or services. These new substances have applications in medicine, the sustainable protection of plants, and industrial biotechnology. This means that insect biotechnology employs the general principles of biotechnology. More specifically, insect biotechnology borrows from and is driven by scientific breakthroughs in molecular biology, particularly by the development of tools and techniques that allow genetic characterization and engineering of organisms and cells as with

recombinant DNA technology and plant protoplast fusion. Recently, bacteria have been engineered to carry and express silk genes from the mulberry silkworm, *Bombyx mori*, and used in the production of synthetic silk, the natural protein fiber, some forms of which can be woven into textiles [8] (see also Chap. 10). As a basic research tool, the strategies of insect biotechnology include the sequencing and annotation of insect genomes as well as analyses using comparative genomics. Insect biotechnological applications to pest management include the development of resistant crops and trees that express insect-specific toxins, the design of microbial agents with enhanced insecticidal potency, and the engineering of insects that can transfer lethal genes to natural populations following their mass release in the field. Comparative genomics analyses also make it possible to identify insect-specific genes that can be targeted for rational insecticide design.

8.2 Applications of Insect Biotechnology in Agriculture

It has been shown by the USDA [9] and numerous other sources that insects and nematodes are the world's most important pests of agricultural plants and livestock. They have been shown to cause billions of dollars of losses to growers and livestock producers every year because of lowered yields, deterioration of quality, and disease. As already indicated earlier in this review, chemical pesticides are a major tool for insect and nematode control. However, safer alternatives to chemical pest control have become imperatives because most of these pesticides are potentially harmful to the ecosystem and human health. The advent of biotechnology in general and insect biotechnology in particular is a welcome development in the fight against insect pests.

Modern agricultural biotechnology or genetic engineering includes manipulation of the genetic makeup of organisms for use in the production or processing of agricultural products. A major strategy in increasing crop yield is the development of pest management regimens consisting of herbicide-resistant (HR) and insect-resistant (IR) crops as well as transgenic insects. Genetically modified (GM) crops first became commercially available in 1996. By 2008, 90 % of soybean, 78 % of cotton, 72 % of canola, and 60 % of maize hectares were cultivated globally with these GM crops [10]. The most well-known example of this genetic manipulation in both plants and viruses is the insertion into a plant or virus of the gene coding for the production of the delta endotoxin of *Bacillus thuringiensis* [11]. *B. thuringiensis* (or Bt) is a Gram-positive, soil-dwelling bacterium, commonly used as a biological pesticide. It also occurs naturally in the environment of mosquito larvae, moth, and butterfly caterpillars, as well as on leaf surfaces, aquatic environments, animal feces, insect-rich environments, flour mills, and granaries [12]. Many Bt strains produce "Cry" during sporulation. Cry (short for crystal proteins; Fig. 8.1), also called δ-endotoxins, are proteinaceous inclusions encoded by plasmid-borne *cry* genes and have insecticidal action on susceptible insects. Bt is remarkably nontoxic to humans, and to a large extent nontarget fauna, and is easy to use, making it a

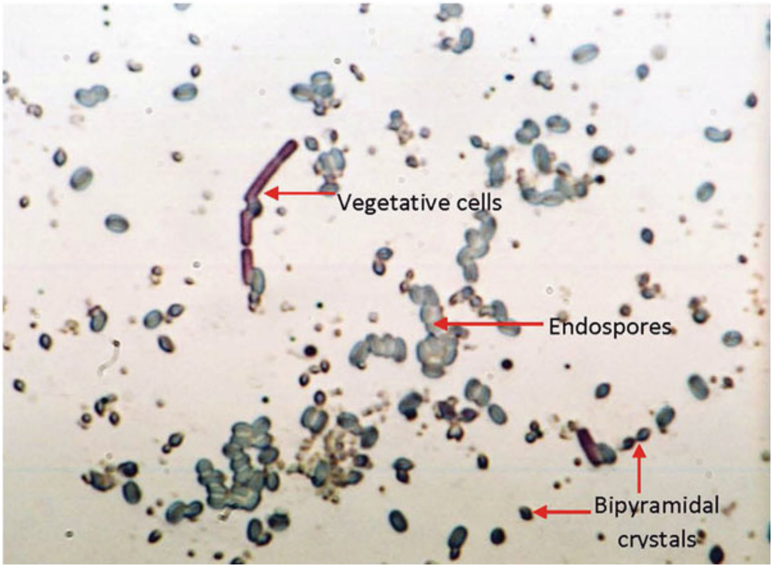


Fig. 8.1 *Bacillus thuringiensis kurstaki* showing crystals, vegetative cells, and spores after staining by a differential crystal staining method. Isolated from soil in Tennessee

popular alternative to chemical treatments for crop protection. This has led to the use of Bt toxins as insecticides and, more recently, to GM crops expressing Bt proteins in transgenic plants.

8.2.1 *About* Bacillus thuringiensis kurstaki

B. thuringiensis (Bt) *kurstaki* has served as a microbial insecticide for many decades, but the widespread use was limited by its instability when exposed to UV radiation under sunlight and its poor retention on plant surfaces in wet weather. The high toxicity of the Bt toxins to a variety of insect pests, and the ease with which the gene could be isolated from bacterial plasmids, made it an obvious choice for the development of the first insect-resistant transgenic plants. The active Bt toxin binds to a receptor in cells lining the insect midgut and creates a channel, allowing free passage of ions. The cells lining the midgut die, and very soon, the insect dies, too. Different strains of Bt contain plasmids encoding different toxins with different specificities of action against insects. A particular toxin is generally effective against only a limited range of closely related species. Bt toxins are used in a variety of transgenic crops, including cotton, for protection against a variety of lepidopteran pests; corn (maize), for protection against the European corn borer, *Ostrinia nubilalis* (Lepidoptera); and potatoes, for protection against the Colorado potato beetle, *Leptinotarsa decemlineata*. Intensive screening programs have led to important

collections of Bt strain isolates in the last few decades [13–18]. This became necessary as reports of the appearance of insect resistance and novel Cry proteins with toxic potential against different organisms emerged together with the need for more potent toxins with specificity for a much broader range of pests. The biosafety and mechanism of Bt toxin action are reviewed elsewhere [19–22].

8.3 Applications of Insect Biotechnology in Medicine

Reports abound on the emergence of new bacterial strains and increasing bacterial resistance against antibiotics leading to a growing number of severe, sometimes life-threatening bacterial infections in hospitals and even in daily life environment. A recent example is the 2011 *Escherichia coli* O104:H4 outbreak in Germany [44]. Reports of this nature clearly demonstrate the urgent need for new antimicrobial drugs. Insects have been shown to produce an array of antimicrobial metabolites which have potential as templates for further drug development.

In 1999, Bulet and colleagues reviewed the arsenal of agents used by insects to fight off invading microorganisms [45]. They reported that it is essentially based on short cationic antibacterial and antifungal peptides/polypeptides (see also Chap. 6). Depending on the species, insect adipose tissue can respond to bacterial infection by the synthesis of a large array of antimicrobial substances (e.g., *Drosophila* adults produce more than ten different molecules, including drosocin, drosomycin, defensin, metchnikowins, diptericin, and cecropins) or by producing relatively few molecules (e.g., a single peptide, defensin, in the dragonfly, *Aeshna cyanea*) [46]. Antimicrobial peptides produced by insects are structurally similar to those produced by many plants and animals. Industry has capitalized on that feature for the development of many new drugs from insects. For example, cecropins [47] have similarities with the amphibian α-helical antimicrobial peptides, magainins, found in frog skin secretions, while drosomycin from *Drosophila* and thanatin from the spined soldier bug, *Podisus maculiventris*, have similarities with plant defensins and frog skin secretion antimicrobial peptides, respectively. Bulet and colleagues [48] concluded that insect antimicrobial peptides are useful in overcoming the problem of acquired bacterial drug resistance and of the emergence of opportunistic pathogens, especially in immunosuppressed hosts due to the fact that:

1. They rapidly kill target microorganisms.
2. They have broad activity spectra.
3. They are not active against mammalian cells.
4. They have a mode of action that should restrict the selection of resistant strains.

A more recent review of insect products and their development as drugs published in 2014 revealed the importance of insect biotechnology in medicine. Based on knowledge gleaned from insect folk medicines, Ratcliffe and colleagues [4] described modern research into bioengineering of honey and venom from bees, silk, cantharidin, antimicrobial peptides, and maggot secretions and anticoagulants from bloodsucking insects into medicines. They described how modern molecular and

Table 8.1 Examples of potential use of products of insect biotechnology in medicine

Product	Source	Uses	Reference
5.8 kDa honey component of manuka honey	*Apis* spp.	Stimulates TNF-α production via TLR4 in human monocyte cultures. For wound repair/healing	[23]
Manuka honey, buckwheat honey, honey products of Revamil, propolis	*Apis* spp.	Inhibits Gram-positive MRSA, vancomycin-sensitive and vancomycin-resistant *Enterococci, Streptococcus* species isolated from wounds, Gram-negative bacteria associated with wounds such as *Pseudomonas aeruginosa*, *Stenotrophomonas* species, and *Acinetobacter baumannii*	[24–29]
Quercetin, apigenin, and acacetin	*Apis* spp.	Anticancer, enhances the apoptotic ability of anti-CD95 and rTRAIL in acute lymphocytic leukemia	[30–32]
Bee, wasp, and ant venom products, e.g., melittin	*Asp* spp., *Vespula* spp., *Solenopsis* spp., *Pachycondyla* spp., and *Myrmecia* spp.	Inhibits or kills cancer cell types including hepatic, melanoma, ovarian, osteosarcoma, leukemic, prostate, mammary gland cells, bladder, and renal	[33–35]
Maggot alloferons	*Lucilia sericata*	Antiviral and antitumor activities	[36]
Cantharidin	Blister beetles, *Mylabris caragnae*, and *Mylabris phalerate*	Treatment of cancers including hepatic, colorectal, melanoma, pancreatic, bladder, breast, and leukemia as well as activities against *Plasmodium falciparum* and *Leishmania major*	[37–41]
Anopheline	*Anopheles* salivary gland	Thrombin inhibition and development of antithrombotics for use as anticoagulants	[42, 43]

biochemical techniques have made it feasible to manipulate and bioengineer insect natural products into modern medicines. A summary of some of the most important compounds and their uses in medicine is presented in Table 8.1.

8.4 The Role of Insect Cell Cultures in Insect Biotechnology

Perhaps one of the areas in which insect biotechnology has made the greatest contribution to basic research is the development of insect cell cultures. In general, the establishment of cell cultures (animals, plants, insects, and fungi) is done by growing cells under conditions suitable for propagating stable, likely clonal, lines of cells harvested from embryos, specific tissues, or whole organisms in a variety of culture media developed for the species under investigation as described in Sect. 5. The justification for this is that it is easier to do experiments on individual cells than in animals, considering that the cell is the basic unit of life. It is also easier to produce large

quantities of metabolites, especially heterologous proteins, from cell cultures than from animals. ICs are also excellent systems for the production and posttranslational modification of large quantities of modified eukaryotic proteins in a short time.

The critical issues that must be considered for the successful establishment and maintenance of insect cell cultures [49, 50] are as follows:

1. The use of actively growing cells (i.e., cells in exponential growth phase) to start the culture. Cells such as the Sf9 and Sf21 may be obtained from the American Type Culture Collection. A temperature of 27–28 °C is optimal for growing these cells. Starter media such as TC-100 medium (Sigma, Cat# T3160) supplemented with heat inactivated 10 % fetal bovine serum (FBS) double in 20–24 h and do not require CO_2. Mammalian sera contain complement which mediates quick inactivation of baculoviruses (BVs). It is important to start cells from a frozen cell stock to avoid contamination. High-quality insect cells are crucial for the success of BV expression. Clumping could be a problem and should be avoided. Avoiding media particles could help reduce clumping, and gentle shaking in shake flasks could help dislodge them.
2. The importance of medium quality cannot be overemphasized. Synthetic serum-free media are available from Invitrogen and other suppliers and are hassle-free. Certain media can be preferred for certain applications. For example, synthetic serum-free media are strongly recommended for the expression of secreted proteins but can be also used for non-secreted protein studies and for transfections.
3. Transition from serum-containing to synthetic serum-free medium (weaning) is done gradually on healthy cells growing in log phase. It is done by slowly decreasing the percentage of serum-containing medium and increasing the percentage of synthetic serum-free medium in the cell culture over a period of about a week.
4. Antibiotics may be used to control contamination. An example is penicillin/streptomycin solution (Invitrogen SKU# 15070–063) used at 1:99 dilution. Gentamycin and nystatin can also be used routinely in insect cell culture. The use of antibiotics is optional.
5. Insect cells such as Sf9 or Sf21 cells can be stored in liquid nitrogen for many years frozen in TC-100 or similar medium supplemented with heat inactivated 10 % FBS. When needed, frozen cells may be thawed in a carefully designed protocol to avoid damage.
6. Cleanliness of glassware is an important requirement. Insect cells are very sensitive to contaminants, especially detergents. Flasks should be washed using ~1 % Triton X-100.

Insect cell cultures are widely applied in a variety of scientific fields. They are also used for the production of recombinant proteins, viral pesticides, and vaccines, as well as for basic research on morphogenesis, genetics, virology, pathology, biochemistry, endocrinology, and molecular biology. Cell lines have been established from several orders of insect including Diptera, Lepidoptera, and Hemiptera. Dipteran and lepidopteran cell lines are particularly important in agriculture and biotechnology and will be discussed in more detail below.

The first breakthrough in cell line development from arthropods was spindle-, round-, and crescent-shaped cells from the ovarian tissues of the Gum Emperor moth, *Antheraea eucalypti* Scott (available as ATCC® CCL-80™), a native of Australia and New Zealand [51]. These were grown on Grace's insect tissue culture medium, which is a modified Wyatt medium [52] supplemented with ten vitamins. This medium gained acceptance in research and commercial applications, not just because it supports the growth of cells in vitro but also because of its usefulness in the establishment and maintenance of many insect cell lines [53, 54]. Since the introduction of Grace's medium, there has been a geometric upsurge in the study of ICs leading to the establishment of over 500 cell lines from different insect species and orders [55–57].

More recently, interest has been focused on developing new lepidopteran cell lines for potential application in biotechnology, largely thanks to advances made in recombinant DNA technology starting in the early 1970s which has led to the production of new recombinant proteins and genes useful in public health and agriculture. Lepidopteran cell lines have been established primarily to propagate insect viruses as biopesticides for the biological control of insect pests [58]. Lately, the baculovirus expression vector system combined with insect cell cultures (IC-BEVS) has become more attractive for the expression of many heterologous proteins than those derived from bacteria, yeast, vertebrates, or viruses due to its unique characteristics. This technology is also being used for the construction of recombinant BVs to use as biopesticides, which offer comparatively faster killing of insect pests than wild-type BVs [59–62].

Insect cell lines have become invaluable in:

(a) The study of virus–cell interactions [63–66]. This has enhanced the application of insect cell lines as a tool in basic research.
(b) Virus-related research for the development of viral pesticides [67, 68]. This is the cornerstone of the application of insect cell lines in agriculture.
(c) The production of recombinant proteins/vaccines [69]. This constitutes the foundation for application of insect cell lines in biotechnology.
(d) The development of health-related products using vectors developed from insect viruses, especially BVs (with the major genus being the nucleopolyhedrosis viruses [NPVs]) [70]. This has defined the application of insect cell lines for the control of vectors of human diseases and has had great impact in health and disease.

8.5 Insect Cell Line Development, Maintenance, and Production

Given the importance of insect cell culture as a foundation for modern insect biotechnology and, importantly, the production of recombinant proteins, the following section will discuss briefly the media and methods of insect culture development, maintenance, and production.

Generally, nutrient requirements of ICs and those of vertebrate cells are essentially similar, although there are a few differences that must be considered when designing insect cell culture media. The following conditions are present in insect hemolymph and must be met for the formulation of successful culture media:

(a) A high level of amino acids [49, 71]
(b) High levels of free organic acids such as citrate, succinate, oxalate, or malate, which range from 0.1 to 30 mmoles per insect [49, 72]
(c) Acidic conditions in a normal range of pH 6.2–pH 6.9, higher than typically found in mammalian tissue fluids [49]
(d) Supplementation with metabolic sources of cell membrane components and the steroid hormone, ecdysone, because ICs lack the capacity to make steroids [49, 73]
(e) Osmolarity in the range of 340–390 mOsmol/kg, more than twice as high as that of the vertebrate blood [74]
(f) High concentrations of metabolites in the glutamine and glucose metabolism pathways [72, 73]

As indicated previously, Grace's medium has always been one of the most popular media for insect cell culture. The formulation was designed to mimic the chemical composition of the hemolymph from *B. mori*. Prior to use, the medium is typically supplemented with fetal calf serum, yeast extract, lactalbumin hydrolysate, and bovine serum albumin in varying combinations and amounts. Since Grace's medium, other media have been developed for specific uses in insect biotechnology. For example, ESF 921 medium was developed to address the need for a single medium that can support high cell growth using Sf9, Sf21, *Trichoplusia ni* (e.g., High Five™ and Tn 368 cells), and *Drosophila* cells (e.g., S2). High levels of recombinant protein have been expressed in ESF 921 using a variety of cell lines with insect cell baculovirus expression vector system (IC-BEVS; see below) technology or the stable transformed cell technology. ESF 921 was designed to especially enhance the expression of glycosylated proteins. Sf9 and Sf21 were developed from *Spodoptera frugiperda* ovarian tissue, while BTI-Tn5B1-4 (High Five™) and S2 were developed from *T. ni* embryonic tissue and *Drosophila*, respectively.

Insect cell lines are typically cultivated in steps depending on the magnitude of ultimate deployment (see Sect. 7.7). The steps include:

(a) Shaking in flasks containing serum-free medium to loosen adherent cells
(b) Growth in 2.5–30 L perfusion bioreactor or up to 200 L (where large scale is needed)
(c) Primary separation as part of downstream processing
(d) In-process analytics
(e) Large-scale protein purification for protein expression experiments

The maintenance of high-quality ICs is absolutely important for the success of the all-important IC-BEVS. Comparatively, mammalian cell lines do not deteriorate as fast as insect cell lines, which calls for strict maintenance. As already indicated, most researchers routinely use *S. frugiperda* Sf9 and Sf21 cell lines (Sf9 is a subclone of Sf21). It is advisable to start cells from a frozen cell stock which needs to be pre-warmed to room temperature. This is to avoid contamination, over-passage

of cells, and negligence in maintenance resulting in slower division with time. It is recommended that pre-warmed cultures be diluted about 1:10 to a density of 1×10^6/ml and grown on TC-100 medium supplemented with 10 % FBS [73–75].

8.6 Development and Application of the Insect Cell Baculovirus Expression Vector System (IC-BEVS)

The commonest type of insect viruses is the BVs. BVs are known to infect over 600 insect species worldwide. Members of 11 families are pathogenic to insects. BVs are usually associated with the insect arthropod orders of Lepidoptera, Hymenoptera, Diptera, Neuroptera, Coleoptera, Trichoptera, Crustacea, and Acarina (mites). The virions are rod shaped, 40–70 nm × 250–400 nm, comprising a lipoprotein envelope surrounding a protein capsid containing a DNA–protein core. NPVs include those that infect the lepidopterans, *Helicoverpa armigera*, *S. litura*, *S. exigua*, *Amsacta moorei*, *Agrotis ipsilon*, *A. segetum*, *Anadividia peponis*, *T. ni*, *Thysanoplusia orichalcea*, *Adisura atkinsoni*, *Plutella xylostella*, *Corcyra cephalonica*, *Mythimna separata*, and *Phthorimaea operculella* [76]. Those that infect caterpillars of lepidopteran insects and dipteran larvae appear to have attracted the greatest attention. Each BV is highly specific in its host range, being limited to a single type of insect. The unique characteristics of BVs have enabled the development of the IC-BEVS, which has found great use in biotechnology. IC-BEVS are safe and lend themselves to abundant and rapid production of recombinant proteins in ICs. The development of the first(?) IC-BEVS is credited to MD Summers, who was granted a patent for it in 1988. The IC-BEVS has been widely used in research and scientific industrial communities for the production of high levels (up to 1000 mg/mL) of correctly posttranslationally modified (folding, disulfide bond formation, oligomerization, glycosylation, acylation, proteolytic cleavage), biologically active, and functional recombinant proteins [77]. The IC-BEVS is based on the introduction of a foreign gene into a region of the genome which is nonessential for viral replication via homologous recombination with a transfer vector containing the target gene. The resulting recombinant BV lacks one of several nonessential genes (*polh*, *v-cath*, *chiA*, etc.) which is replaced with a foreign gene encoding a heterologous protein which can be expressed in cultured ICs and insect larvae.

Because of their capacity to produce many recombinant proteins at high levels and also provide significant eukaryotic protein processing capabilities, IC-BEVSs have extended the frontiers of basic research. Furthermore, important technological advances over the past 20 years have improved upon the original methods developed for the isolation of BV expression vectors. Today, virtually any investigator with basic molecular biology training can isolate a recombinant BV vector relatively quickly and efficiently and use it to produce a desired protein in an insect cell culture [78]. The IC-BEVS has become a core technology for:

- The cloning and expression of genes for the study of protein structure, processing, and function
- The production of biochemical reagents

- The study of regulation of gene expression
- The commercial exploration, development, and production of vaccines, therapeutics, and diagnostics
- Drug discovery research
- The exploration and development of safer, more selective, and environmentally compatible biopesticides consistent with sustainable agriculture

The most widely used lepidopteran cells for IC-BEVS are Sf9 and Sf21 cell lines and the High Five™ cell line, designated BTI-Tn5B1-4. Sf9 cells are a subclone of the Sf21 cells and were selected for their faster growth rate and higher cell densities than the Sf21 cells; both are preferred for virus expansion. Sf21 cells can compare favorably, in terms of heterologous protein expression, to both High Five™ and Sf9 cell lines in certain situations. Figure 8.2 presents a general scheme for BEVS development [73, 79, 80].

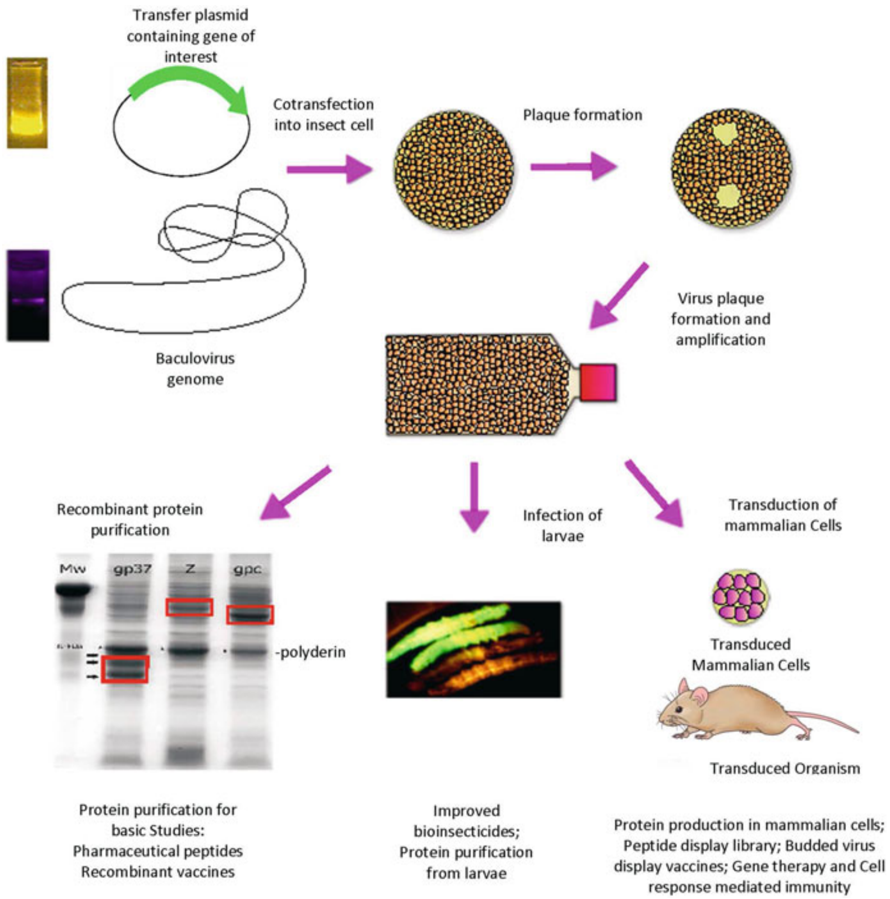


Fig. 8.2 General process for baculovirus cloning. Genomic DNA and a transfer plasmid are cotransfected into an insect cell culture. Recombinant virus propagates [73]

8.7 Applications of IC-BEVS in Industrial Processes

The BV vector most commonly used in industry and research laboratories for recombinant protein production is based on the *Autographa californica* multinuclear polyhedrosis virus (AcMNPV) with Sf9, Sf21, or High Five™ cells; whole *T. ni* insect larvae are also used as suitable expression hosts [79–81].

In recent times, IC-BEVS has also gained prominence and wide acceptance for routine production of recombinant proteins in ICs and larvae [82–86]. Furthermore, IC-BEVS is applied in the development of strategies for displaying foreign peptides and proteins on virus particles and the insertion of mammalian cell-active expression cassettes in BVs to express genes efficiently into many different mammalian cell types. BVs engineered to display foreign peptides and proteins on the viral surface have proven particularly useful as immunogens [87]. The Sf9 and BTI-Tn5B1-4 cell lines described previously, as well as those trademarked by Invitrogen as High Five™ cells, are among the flagship cell lines for producing viruslike particle (VLP) vaccines with recombinant IC-BEVS. VLPs are multi-protein structures that mimic the organization and conformation of authentic native viruses but lack the viral genome, potentially yielding safer and cheaper vaccine candidates [88]. Insect biotechnology, through the development of IC-BEVS, has really revolutionized health and biotechnology industry, as shown in Fig. 8.3 and described in subsequent sections.

8.7.1 Vaccines and Vaccination

Two animal vaccines are presently on the market, and several immunotherapeutic and human vaccines are being developed and produced in Sf21, Sf9, expresSF+™, or High Five™ cells. In Europe, two commercial subunit vaccines for classical swine fever are produced in *S. frugiperda* cells by Intervet, Leiden, The Netherlands [89]. Several human vaccines are being produced in *S. frugiperda.* They include:

(a) Provenge® (sipuleucel-T), an immunotherapeutic vaccine for prostate cancer developed by Dendreon Inc, Seattle, WA
(b) Flublok®, a vaccine for human influenza virus developed by Protein Sciences Inc., Meriden, CT
(c) Chimigen® vaccines for chronic hepatitis B and C developed by ViRexx Medical Corp., Calgary, Canada

Commercial products developed using *T. ni* High Five© cells include:

(a) FavId® (idiotype/KLH), an immunotherapeutic vaccine for B-cell non-Hodgkin's lymphoma developed by Favrille Inc., San Diego, CA.
(b) Cervarix®, a vaccine for cervical cancer developed jointly by MedImmune, Gaithersburg, MD, and GlaxoSmithKline, Rixensart, Belgium. Cervarix is the first vaccine produced in ICs to be commercialized for human use.

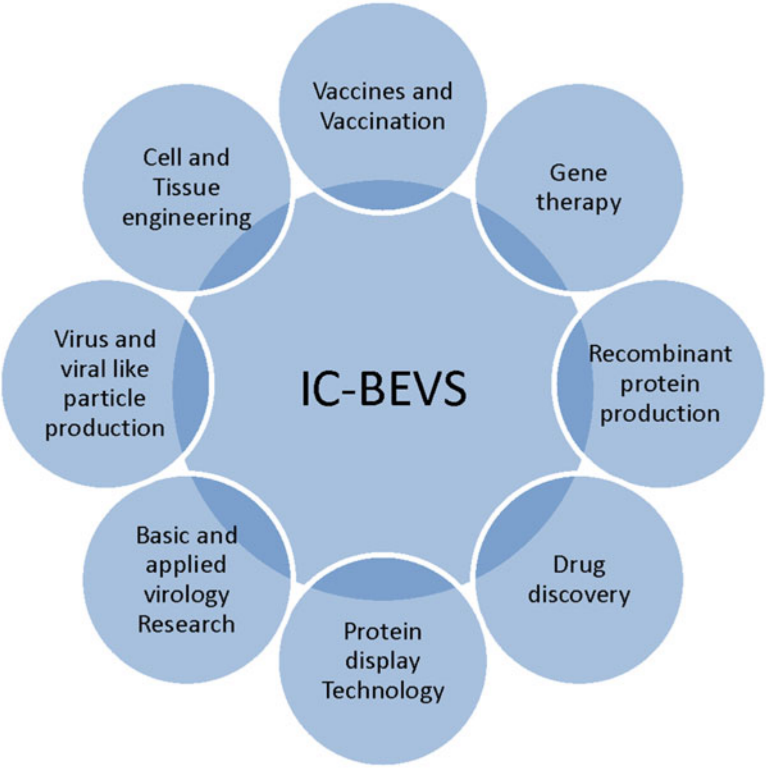


Fig. 8.3 Applications of insect cell baculovirus expression vector systems (IC-BEVS)

The commercial availability and probable success of vaccines for animal or human use in the near future will provide even greater impetus for the application of the BV-insect cell technology in research and medicine.

8.7.2 Gene Therapy

The pioneering studies of Hofmann [90] and Boyce [91] and their coworkers in the mid-1990s, in which they outfitted AcMNPV with an expression cassette driving a marker gene via a strong viral promoter (active in the target cells), boosted interest in BVs as potential vectors for gene therapy. A well-known and accepted economic process for this purpose is the production of recombinant adeno-associated virus (rAAV) vectors in lepidopteran Sf9 cells using recombinant AcMNPV or other BVs [92–96]. BV-mediated gene transfer has already demonstrated therapeutic efficacy in ex vivo and in vivo gene therapy studies [97–100]. Gene therapy has so fully come of age so much that the high potential of this therapeutic modality can now be

realized for treating several diseases, such as severe immune deficiencies [101, 102], ocular diseases [103], and cancer [104]. Several virus-derived vectors and non-virus gene transfer agents have been used to address these applications. Lentiviruses (LVs) have become widely used for gene delivery and gene therapy when sustained gene expression is required [105]. The application of BV for producing AAV and LV facilitates the development of the gene therapy field from the bioprocess perspective. Currently, BV transduction has been used to generate stem cells carrying therapeutic payloads of interest suitable for cartilage and bone tissue engineering and targeted glioma gene therapy [106]. Effective transduction and high-level transgene expression mediated by baculoviral vectors are especially suited for cancer gene therapy [107]. Baculoviral vectors armed with suicide genes, tumor suppressor genes, proapoptotic genes, immunopotentiating genes, and anti-angiogenesis genes have been tested in vivo in animal tumor models in many different anticancer regimens [108–110]. More recently, ex vivo gene therapy studies have been conducted using BVs equipped with suicide genes to transduce stem cells [112–114]. This approach benefits from the tropism exhibited by many adult stem cells for primary, solid, and metastatic tumors. Kotin and coworkers have extended the versatility of the IC-BEVS to include the production of faithfully assembled and packaged rAAV vectors suitable for in vivo gene therapy applications [115].

8.7.3 Recombinant Protein Production

As already stated, the IC-BEVS is one of the most powerful, robust, and versatile eukaryotic expression systems available [116]. Given its speed of development and versatility for the expression of a wide range of protein families, the IC-BEVS offers multiple advantages for protein production in a variety of applications. Since the development of the IC-BEVS in the 1980s [117], thousands of recombinant proteins, ranging from cytosolic enzymes to membrane-bound proteins, have been successfully produced in BV-infected ICs. However, expression yields in optimized transformed fed-batch mammalian cells cultured in a bioreactor commonly reach grams of recombinant protein per L; in ICs infected by recombinant BVs, the yield rarely exceeds 50–100 mg per L. This relatively low expression capacity can be compensated in the IC-BEVS by the short development times and lower costs associated with a specific product. This implies that for the production of a recombinant protein with market needs not exceeding 5–10 kg per year (i.e., subunit vaccines), the IC-BEVS is currently one of the best alternatives [118]. In fact, most licensed products obtained in ICs correspond to vaccines and not to products with a high production demand such as therapeutic antibodies [119]. However, the yield is not the only bottleneck of the IC-BEVS. A marked proteolysis of recombinant proteins during BV-based production is frequently encountered. This observation is due, in part, to the cytopathogenic effects of the BV vectors in ICs during infection [69, 93, 94].

The in-depth understanding of both uninfected and infected insect cell physiology using metabolic engineering and the constant improvement of insect cell media and culture techniques are bound to contribute to the rational design and validation of robust, scalable, and safe production processes leading to improved yields based on the IC-BEVS technology.

8.7.4 Drug Discovery

Information abounds in the scientific and biotechnology literature about significant advances in BEVS technology which has led to the production of more than 1000 viral, prokaryotic and eukaryotic recombinant proteins. Many biotechnology initiatives and start-up industries have arisen in the USA and the rest of the industrialized world based on the BEVS and its applications for discovery, development, and commercial production areas for medicine and agriculture [85]. To achieve the desired success, drug and vaccine discovery must take into account that large amounts of complex eukaryotic proteins are produced with appropriate posttranslational processing and have desired biological activity. Fortunately, BEVS provide the platform to achieve this cheaply and fast. The phenomenal applicability of BEVS technology in drug discovery has been accentuated by the ability to crystallize medically important proteins, to advance the understanding of three-dimensional structure, and to enhance the precise design of drugs that are specifically targeted for specific intervention in many diseases.

Examples of the application, efficiency, and cost-effective BEVS technology in the rapid development of drugs include an experimental vaccine for the deadly Hong Kong "bird flu" virus (H5N1). The first of this vaccine, Pandemrix, was approved in 2013 by the US Food and Drug Administration and rapidly manufactured by GlaxoSmithKline PLC (GSK) [95]. Currently, no definitive medication protocol specific to severe acute respiratory syndrome (SARS) has been developed; however, there has been a rapid development of a test SARS vaccine upon request by the US National Institutes of Health. Protein Sciences Corporation (PSC) used BEVS technology to develop and deliver 1700 doses of an experimental "bird flu" vaccine in 2 months. PSC has also developed Flublok®, the world's first recombinant protein-based seasonal influenza vaccine based on the BEVS technology [96].

In 2013, 30 years after its inception, the baculovirus–insect cell expression system and its associated technologies were celebrated. This system still forms a mainstream platform for the production of recombinant proteins for fundamental and applied science. Scientists are encouraged to take this system further into the next decades by overcoming remaining challenges, e.g., to optimize BEV genome composition, to improve genome stability in order to guarantee product quality over batches, and to simplify downstream processing without losing safety. This will allow the baculovirus expression system to become the system of choice for many applications based on its convenience and the yield, quality, and safety of the recombinant products [97].

8.7.5 Protein Display

In addition to the numerous advantages and usefulness of IC-BEVS already discussed, they have become one of the systems of choice to display vectors that would be suitable for targeting and gene transfer to mammalian cells. Display of heterologous proteins is usually carried out using mammalian viruses such as retro-, adeno-, and adeno-associated viruses [98, 85]. The main advantage of molecular display technology is the physical linkage between the proteins or peptides displayed (phenotype) and the genes that encode them (genotype). This allows simultaneous selection of the genes with proteins of the desired affinity or function [99]. Among extremely versatile display technologies are those based on the IC-BEVS. The expression of foreign proteins on the BV surface was described previously. The applicability of IC-BEVS display technology in the generation and screening of eukaryotic expression libraries has been eminently demonstrated and established [100, 101]. The first proteins chosen to be displayed were glutathione S-transferase and the external domain of gp120, a surface glycoprotein of human immunodeficiency virus type I (HIV-1). Both were successfully expressed on the BV 25 surface as N-terminal fusions to the mature gp64 protein; additionally, gp120 was shown to be functional in binding to its ligand, CD4 [102]. Variations of the HIV-1 gp41 epitope, "ELDKWA," were BV-infected ICs and screened with a specific neutralizing monoclonal antibody. In 1997, Grabherr and colleagues demonstrated the presentation of the ectodomain of the HIV-1 gp41 envelope protein on the virus surface, either as a fusion to the entire gp64 or, alternatively, to its transmembrane domain [101, 100]. Other BEVS-based systems include:

1. Immunization of mice with recombinant baculovirus (rBV) displaying gp64 amino-terminal fusion proteins for the nuclear receptors, liver X receptors (LXRs) and farnesoid X-activated receptor (FXR). This is used to develop monoclonal antibodies to LXR and FXR. LXRs are ligand-activated transcription factors of the nuclear receptor superfamily. LXR activation normalizes glycemia and improves insulin sensitivity in rodent models of type 2 diabetes and insulin resistance. FXR is a member of the nuclear receptor superfamily. It has emerged as a key player in the control of multiple metabolic pathways. On its activation by bile acids, FXR regulates bile acid synthesis, conjugation, and transport, as well as various aspects of lipid and glucose metabolism.
2. Display of the green fluorescent protein (GFP) and envelope glycoproteins E1 and E2 of rubella virus on the BV surface [103, 104].
3. The use of BV surface display for the presentation of antigenic sites from the foot-and-mouth disease virus [104] and the p67 antigen of *Theileria parva*, the causative agent of the cattle disease East Coast fever [105].
4. The use of a display strategy in which enhanced green fluorescent protein (EGFP) was fused with either the N- or C-terminus of AcMNPV major capsid protein vp39, leading to the high-level incorporation of the EGFP–vp39 fusion into the BV capsid with apparent compatibility with oligomeric proteins [106, 107].

8.7.6 IC-BEVS in Basic Virology Research

The field insect biotechnology based on cell lines and IC-BEVS has grown to the extent that now over 500 insect cell lines have been established from many insect species representing numerous insect orders and from several different tissue sources. These systems are used as research tools in virology, to study signaling mechanisms in insect immunity and hemocyte migration and to test the hypotheses about gene expression, and in screening programs designed to discover the mode of action of new insecticides. Such virology research is revealing fundamentally new information on virus/host cell interactions and uncovering signal transduction pathways that are new to insect science. Research based on IC-BEVS is leading to the development of high-speed screening technologies which are essential in the search for new insect pest management tools. In addition to the use of a few insect cell lines designed to produce proteins of biomedical significance in routine industrial processes, as described previously, both primary cell cultures and established lines are used in basic biological studies to reveal how ICs work.

The continued development of new cell culture technology is essential for the continuously growing application of BV biotechnology. Cell lines used for academic research are currently dominated by the same ones used for commercial purposes described earlier, namely, the *S. frugiperda* line, Sf21, and its clonal isolate, Sf9, and the *T. ni* line, BTI-Tn5B1-4 or High Five™ cells, thus, the long-held prediction that the immense potential application of the IC-BVS as a tool in cell and molecular biology, agriculture, and animal health has been achieved [108, 85]. The versatility and recent applications of this popular expression system have been demonstrated by both academia and industry, and it is clear that this cell-based system has been widely accepted for biotechnological applications. Numerous small to midsize start-up biotechnology companies in North America and Europe are currently using IC-BEVS technology to produce custom recombinant proteins for research and commercial applications. The recent breakthroughs in the development of several commercial products that will impact animal and human health, as noted above, will further enhance interest in this technology for pharmaceutical applications [108, 85].

8.7.7 Virus and Viruslike Particle Production

As noted previously, the ability to make a large variety of VLPs has been successfully achieved in the IC-BEVS/insect cell system [109]. The production and scale-up of these particles are currently being addressed, mostly for candidate vaccines and as delivery agents for use as therapeutics. Recently, as noted, AAV vectors, which can be potentially used for human gene therapy, have been produced in ICs using three BV vectors to supply the required genes. The use of the host IC allows mass production of VLPs in a proven scalable system. This chapter focuses on the

methodology, based on the work done by various laboratories, for the production of AAV-like particles and vectors in a BEVS/insect cell system [110]. New BV–AAV reagents have made it possible to produce rAAV using two or three different BVs. Using this multiplicity of infection (MOI), strategy improves the chances of producing a reengineered two-BV system in benchtop-scale production. The percentages of rAAV producer cells increase at higher MOIs. Production of the reengineered cells can also be done at pilot/large scales with MOIs of ≤1. The downstream processing methods (not discussed in this section) depend on the scale of production.

8.7.7.1 Benchtop-Scale Production

Sf9 cells are grown in benchtop shaker incubators at 27–28 °C with agitation to provide sufficient dissolved O_2 in the medium. This is needed to maintain the cells in exponential growth phase which is ideal for rAAV production. Small culture-volume rAAV preparations are routinely performed by either triple infection (Rep baculovirus, Cap baculovirus, and vector genome baculovirus) or double infection (Rep-Cap baculovirus plus the vector genome baculovirus). AAV vector may be harvested directly from the shake flask when cell viability decreases to <30 % by freeze–thaw lysis. The cell lysate may then be nuclease-treated to reduce viscosity and digest extravirion DNA. Debris is removed by centrifugation or filtration and the rAAV is concentrated by adding polyethylene glycol (PEG, 8000 MW) to the clarified lysate (2 %, w/v) and enhancing the precipitation of the rAAV particles [70, 109, 110].

8.7.7.2 Pilot- or Large-Scale Production

Production of IC-BEVS at pilot or large scale is done in stirred tank bioreactors and can only be limited by the capacity of the available upstream and downstream processing systems. Commercially available media include serum-free formulations from several manufacturers. As in the benchtop scale, consistently regulated temperature, agitation, and dissolved oxygen are the most critical parameters for promoting cell growth and rAAV productivity. The buffering capacity of these commercial growth media adequately maintains the acidity of the culture within levels acceptable for the growth of Sf9 cells, making pH monitoring optional. The low-MOI strategy used for heterologous protein expression in the baculovirus–insect cell system has proved very useful for rAAV production [111, 112]. Negrete and colleagues [76, 77] found that using MOIs much less than 1 was effective for rAAV production if the cell density at the time of infection was appropriately adjusted to accommodate the increased time until baculovirus-induced cell cycle arrest. Initially, few cells are infected, and the probability that different viruses coinfect a cell is near zero. Thus, using a primary MOI of 0.05, the MOI of the secondary round is about 5 [113, 114].

8.7.8 *Use of Baculoviruses in Cell Therapy and Tissue Engineering*

Tissue engineering is the use of a combination of cells, engineering, and material methods, together with suitable biochemical and physicochemical factors, to improve or replace biological functions. While it was once categorized as a subfield of biomaterials science, having grown in scope and importance, it can be considered as a field in its own right. The term is closely associated with applications for repairing or replacing portions or whole tissues (i.e., the bone, cartilage, blood vessels, bladder, skin, muscle, etc.). Great success has been achieved in the area of bone marrow mesenchymal stem cells which are considered a highly potent vehicle for cell-based therapies [115] due to their differentiation potential and special immunological properties such as suppression of the host graft response. BV transduction of mammalian cells does not seem to seriously compromise their immunological status and behavior in vitro and, more importantly, in vivo [116, 117]. BVs are potentially safe candidates for therapeutic gene delivery because of their intrinsic inability to replicate in mammalian cells and their low cytotoxicity [53, 68, 118–120]. Articular cartilage is a heavy tissue that protects bones, but is limited in self-repair capacity. Hu and coworkers first demonstrated that BV can effectively transduce rat articular chondrocytes [86, 121–123]. However, chondrocytes tend to dedifferentiate during subculture, thus hindering their in vitro expansion and subsequent transplantation back to the host. Sung and colleagues demonstrated that transduction of dedifferentiated chondrocytes with a BV vector (Bac-CB) expressing bone morphogenetic protein 2 (BMP-2) not only restores the differentiation status of passaged chondrocytes in vitro but also increases accumulation of cartilage-specific extracellular matrix [124]. These activities were further augmented by BV-mediated coexpression of transforming growth factor β [125, 126]. Lin and colleagues recently constructed a recombinant BV (Bac-CV) expressing vascular endothelial growth factor (VEGF) [82, 127, 128]. Implantation of Bac-CV- and Bac-CB-transduced rabbit bone marrow-derived mesenchymal stem cells into critical-size segmental bone defects at the femora of New Zealand White (NZW) rabbits accelerated and ameliorated bone healing, thanks to the in vivo coexpression of *BMP-2/VEGF*, ensuing improved osteogenesis and angiogenesis [129–131].

8.8 Conclusion

The production of bioinsecticides and recombinant proteins for use in basic research, diagnostics, and biomanufacturing are well established and are among the numerous benefits of insect biotechnology to mankind, especially propelled by IC-BEVS. As a result of new applications of the technology in human and animal health, including biopharmaceuticals and new-generation vaccines, the IC-BEVS platform has become the technology of choice for virologists worldwide. The technology is also

seamlessly suited for producing viral vectors for cell therapy and for readily expressing proteins of interest in fundamental biological research, tissue engineering, drug discovery, sustainable agriculture, and other applications. The continual improvement of tissue culture techniques that promote high-quality cell systems as well as the use of BV as a display system is building on the improvement of the quality of insect cell media and efficiency of cultivation. Generally, insect biotechnology is a most auspicious development in modern scientific and biotechnological pursuits leading to scalable and safe IC-BEVS-dependent biomanufacturing processes.

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Chapter 9
Spider Silks and Their Biotechnological Applications

Daniela Matias de C. Bittencourt

Abstract Spiders have developed specialized silks with outstanding biophysical properties over millions of years of evolution. As biopolymers composed by highly repetitive amino acid motifs, spider silks have been the focus of research for years. Due to recent advances in genetic engineering, recombinant spider silks have been produced, revealing the relationships between their protein structure and their mechanical properties. Each amino acid motif present in the silk adopts a particular secondary structure responsible for conferring a specific mechanical property to it. This feature has opened up the possibility to produce recombinant silks with controlled properties for various biotechnological applications. Moreover, spider silks are biocompatible and biodegradable biomaterials, which also allow their application in medicine. Accordingly, the relationship between molecular composition, secondary structure, and mechanical properties of spider silks is described in this chapter, along with a discussion of the current strategies for the production of recombinant spider silks, their importance in new biotechnological applications, and the current status of the field.

Abbreviations

A	Amino acid alanine
CaMV	Cauliflower mosaic virus
EGFP	Enhanced green fluorescent protein
ER	Endoplasmic reticulum
Flag	Flagelliform spidroin
G	Amino acid glycine
HFIP	Hexafluoroisopropanol
MaSp1	Major ampullate spidroin 1

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MaSp2	Major ampullate spidroin 2
MiSp	Minor ampullate spidroin
P	Amino acid proline
T-RNA	Transfer ribonucleic acid

9.1 Introduction

Spiders have developed specialized silks with outstanding biophysical properties over millions of years of evolution [1–3]. They are capable of producing the most architecturally complex spider webs possible, which are able to stop the tremendous kinetic energy of flying insect prey. Orb-weaving spiders (Araneomorphae: Orbiculariae) spin silks that are mechanically superior to most synthetic or natural high-performance fibers [4]. Spiders can possess up to seven morphologically distinct pairs of spinning glands; this allows them to produce fibers from an aqueous silk dope solution, composed basically of high molecular weight proteins, at ambient temperature and pressure [5–7]. With the exception of the aggregate gland, which is responsible for the production of the aqueous coat present on the spiral threads in orb webs, the major and minor ampullate, flagelliform, tubuliform, pyriform, and aciniform glands are responsible for the production of a single kind of silk perfectly adapted for the specific requirements demanded by the spider's lifestyle and habitat (Fig. 9.1) [8].

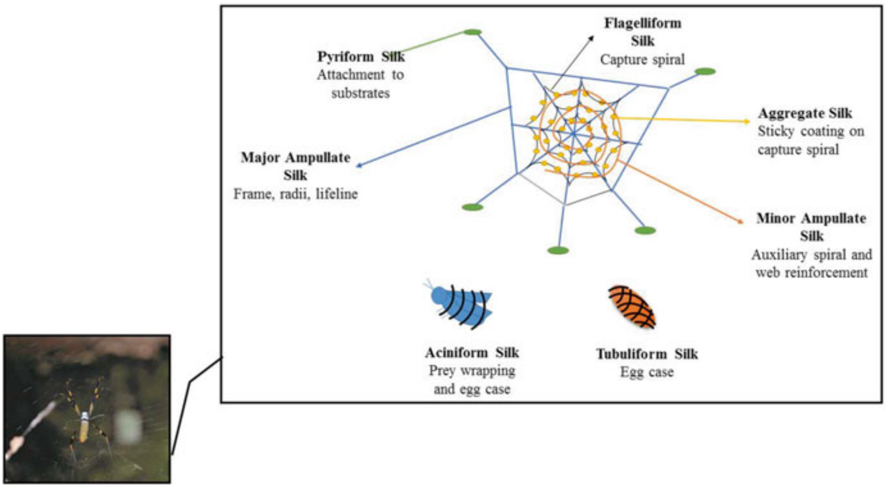


Fig. 9.1 An adult female orb-weaver spider, *Nephila clavipes*, and her silks. Each type of silk has evolved with a unique set of properties needed for a specific application. Portions of the figure were adapted from [50, 73] and reprinted with permission

At the molecular level, spider silk proteins (spidroins) consist of the repetition of different structural amino acid motifs, which are ultimately responsible for the mechanical properties of the silk fibers [2, 9–11]. These motifs vary according to the type of the silk and are normally present at the central core of the protein flanked by non-repetitive amino (N–) and carboxyl (C–) termini regions. This feature, together with biocompatibility [12, 13], low inflammatory potential, antimicrobial activity [14], and a slow rate of degradation [15], has opened up the possibility to produce recombinant silks with controlled properties for various biotechnological applications, from ballistic vests and sporting goods to regenerative medicine [16–18].

Advances in genetic engineering have led several laboratories to successfully produce synthetic spider silks from different heterologous host systems [19–24]. However, the mechanical properties of natural spider silk are still unmatched and continue to be the most significant challenge. Accordingly, in this chapter we aim to describe the relationship between molecular composition, secondary structure, and mechanical properties of spider silks, along with a discussion of the current strategies for the production of recombinant spider silks and their importance in new biotechnological applications.

9.2 Molecular Properties of Spider Silks

Spiders are outstanding silk producers. One spider can produce up to six different types of silk, mainly composed of four amino acid motifs: polyalanine (A_n), alternating glycine and alanine (*GA*)*n*, amino acid triplets composed of two glycines and a third variable amino acid (*GGX*)*n*, and glycine-proline-glycine modules (*GPGXX*)*n*. Each motif is responsible for the adoption of a specific secondary structure in the protein, and the different combinations will determine the complex array of the mechanical properties exhibited by each silk [9, 10].

Among the different silks produced by spiders, dragline and flagelliform silks are the best characterized because of their remarkable mechanical properties. Being so, this chapter will focus on the characterization of these two spidroins. The function and structure of other silk types are reviewed elsewhere [1, 25, 26].

9.2.1 Dragline Silks

Dragline silk forms the frame and radii of the web and serves as a lifeline for the spider while moving through the environment. Produced by the major ampullate gland, dragline silks are known for their high tensile strength and toughness, with strength values in the range of 1–2 GPa [10, 27, 28]. Table 9.1 compares the material properties of some dragline silks from different spider species. The dragline silk

Table 9.1 Comparison of mechanical properties of forcibly silked major ampullate silks from different spider species[a]

Species	Stiffness (GPa)	Strength (Mpa)	Extensibility (mm/mm)	Toughness (MJ/m^3)
Nephila clavipes	13.8	1215	0.172	111
Argiope trifasciata	9.2	1137	0.215	115
Latrodectus hesperus	9.5	959	0.224	132
Deinopis spinosa	13.5	1329	0.185	136
Caerostris darwini	11.5	1652	0.52	350

[a]Adapted from Agnarsson et al. [29]

produced by Darwin's bark spider (*Caerostris darwini*), which is about five times higher than Kevlar, is the toughest silk fiber measured to date (250 MJ/m^3) [29].

Dragline is composed of two different spidroins with molecular size of more than 300 kDa – major ampullate spidroin 1 and 2 (MaSp1 and MaSp2) [30]. The repetitive core region of MaSp1 is formed by the repetition of *A*n, (*GA*)n, and (*GGX*)*n* (X = Y, L, and Q) motifs, flanked by non-repetitive domains at the N– and C– terminal ends. Although MaSp1 and MaSp2 share common features, the latter also has a high proline content, reflecting the fact that (*GGX*)*n* motifs are alternated with (*GPGXX*)n repeats (X = Q, G,Y) (Fig. 9.2). Each of these motifs is responsible for the three-dimensional structure of the protein, which determines the mechanical properties exhibited by dragline silks. The *An* and (*GA*)n motifs are responsible for fiber toughness due to the adoption of β-sheet structures which assemble into crystalline substructures in the thread [9]. Surrounding these crystalline structures is an amorphous matrix composed of (*GGX*)*n* and the (*GPGXX*)n motifs from MaSp2 which is responsible for the elasticity of dragline silk [31]. These regions, which are rich in glycine, include helix-like structures and β-turns, whereby the GGX helices act as cross-links between the crystalline β-sheets in adjacent protein molecules, facilitating fiber alignment [10]. Additionally, dragline silk has the ability to undergo supercontraction in contact with water or a relative humidity greater than 60 %. This feature is probably controlled by the proline content, which tunes the mechanical behavior by changing the crystal cross-link density and hydrogen-bonding network [32, 33].

Once the silk amino acid content has been directly associated with the mechanical properties of the thread, the performance of the dragline silk depends on the ratio between the two protein components [34, 35]. By calculating the number of proline residues, it is possible to estimate the fiber content of MaSp1 and 2, which varies among spider species. The dragline silk from *Nephila clavipes*, for instance, is composed of approximately 80 % MaSp1 and 20 % MaSp2. However, the dragline silk of the orb-weaver spider, *Argiope aurantia*, contains 41 % MaSp1 and 59 % MaSp2. Stress–strain curves determined by polynomial regression show significant differences between major ampullate silk fibers from *N. clavipes* and *A. aurantia*. The *N. clavipes* stress–strain curve is characteristic of an elastic material; in contrast, *A. aurantia* dragline silk has the behavior of a viscoelastic composite [36].

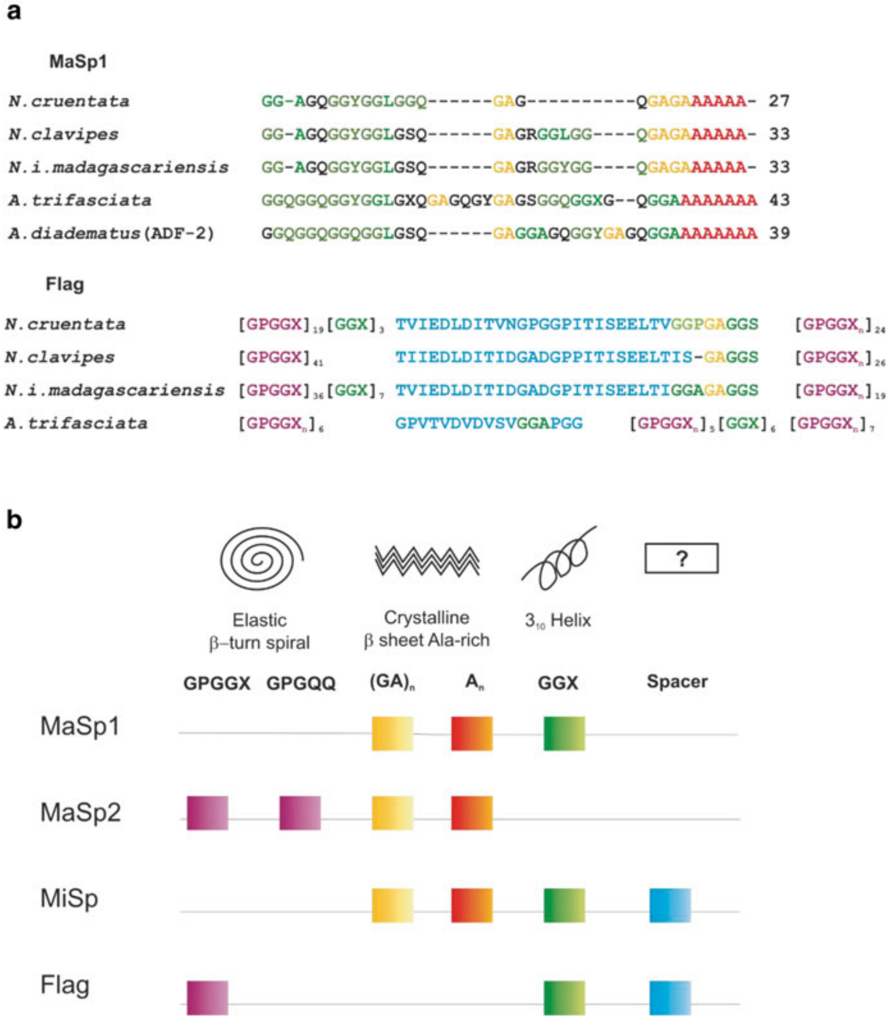


Fig. 9.2 The molecular structure of orb-weaver spider spidroins. (**a**) Alignment of the consensus repetitive sequences of MaSp1 and Flag spidroins. (**b**) Structural motifs found in spider silk proteins. Each *box* represents a motif module in the spider silk. The empty box marked with "?" indicates that the secondary structure is unknown. MaSp1 and 2, major ampullate spidroin; MiSp, Minor ampullate spidroin; Flag, flagelliform spidroin (Reproduced with permission from Teulé et al. [56])

In addition to the repetitive domains, dragline silks contain non-repetitive N- and C-terminal ends. The C-terminal region is highly conserved between species and is essential for controlled switching between the storage and assembly forms of silk proteins [37]. However, while the C-terminal domain is a prerequisite for the formation of continuous silk fibers as opposed to amorphous molecular aggregates, it is the N-terminal domain that senses the pH differences in the glandular lumen and thereby appears to regulate the assembly process in vivo [38].

9.2.2 *Flagelliform Silk*

Among the different silks produced by spiders, the flagelliform silk (Flag) is extremely elastic and has a very high hysteresis, being three times tougher than Kevlar [2, 3, 39]. It is responsible for the formation of the capture spiral in the orb web, an effective energy-absorbing trap for flying prey. Single molecules of the capture silk, Flag spidroins, are very large in size, measuring approximately 360 kDa. Like other elastomeric proteins, such as elastin and abductin, Flag is also characterized by a high content of glycine residues [40]. Its amino acid content is organized into a large ensemble of repeats composed of the individual repetitive motifs, (*GPGGX*)n (X represents A, V, S, and Y) and *GGX* (X represents A, S, and T), and a "spacer" sequence containing charged and hydrophilic amino acids [39, 41, 42] (Fig. 9.2). The glycine-rich central Flag core is also flanked by conserved non-repetitive N- and C-termini. Like in dragline silk, these regions are suggested to control solubility and fiber formation in native spider silk.

The elastic nature of flagelliform silk is conferred by the amino acid motif *GPGGX*. This motif has been suggested to conform to a β-spiral secondary structure similar to a spring, which could easily contribute to the elastic mechanism of the fiber, whereby the proline bonds generate force for the retraction of the silk after stretching [9]. For instance, dragline silk has 35 % elasticity, with an average of five β-turns in a row, whereas flagelliform silk, with >200 % elasticity, has this same module repeated ~50 times [43]. The glycoprotein "glue" coating the capture silk also contributes to the extensibility of the thread. Like MaSp2, Flag has the ability to contract from its original length in contact with water. The hygroscopic components present in the glycoprotein glue attract water from the environment, keeping the web hydrated and thus playing a critical role in the mechanical properties of flagelliform silk fibers [44, 45].

Finally, the spacer regions found in Flag contain amino acids with charged groups and separate the peptide motifs into clusters. Although these may provide organizational areas within silks or surface regions for interactions when a mature fiber is formed, their real function is currently undetermined [9]. Recently, Adrianos and colleagues [11] investigated the contribution of various motifs to the mechanical properties of Flag spidroin using recombinant silk gene expression. Their results indicate that the spacer motif is likely a primary contributor to silk strength, with the GGX motif supplying mobility to the protein network of native flagelliform silk fibers.

9.3 Recombinant Spider Silk Proteins and Their Application

Since ancient times, spider silks have fascinated humankind because of their great mechanical characteristics of strength, toughness, and elasticity. In addition, spider silks are biocompatible and biodegradable, which make them excellent material for

biomedical applications. Due to these characteristics spider silks can be used to make such diverse products as parachute cords, bulletproof vests, composite material for aircrafts, products used in cosmetics, bandages to promote wound healing, drug delivery systems, and scaffolds for tissue engineering [46–49].

Despite the biotechnological potential of spider silks, there are limitations in harvesting large quantities of silk from spiders. They cannot be farmed as for the silkworm (*Bombyx mori*) owing to their aggressive behavior and territorial nature. In addition, silk must be harvested manually, directly from a spider or collected from the web, both extremely time-consuming procedures. Recently, a golden color spider silk cape was made in Madagascar from 1.2 million golden orb spiders (genus *Nephila*) which took 8 years to collect [50]. Thus, new strategies based on genetic engineering have been developed for the production of new biomaterials based on the molecular structure of spider silks.

9.3.1 Production of Recombinant Silk Proteins

Several heterologous host systems have been used for the production of different spider silk proteins [50, 51]. The most widely used host is the gram-negative enterobacterium, *Escherichia coli*, which offers a well-controlled, cost-efficient system suitable for large-scale production. However, spider silk proteins are rather difficult to produce, mainly because of the highly repetitive nature of the nucleotide sequence and the high content of specific amino acids, mainly alanine and glycine, which require a large pool of the respective tRNAs [47, 50]. For this reason, attempts to produce major ampullate silks based on the native DNA sequence in *E. coli* resulted in low protein yields and instability of the spider silk gene, which led to the production of limited size proteins [19]. As an alternative, the methylotrophic yeast, *Pichia pastoris*, was used and was able to produce and secrete recombinant silk proteins up to 65 kDa to the extracellular medium [20]. Mammalian cell lines have also been investigated as a potential host system [21]. Although they were capable of producing spider silk proteins with molecular size ranging from 60 to 140 kDa, as the size of the recombinant silk gene increased, translational pausing occurred, resulting in the expression of heterogeneous size proteins and a decrease in the protein yield.

In order to optimize protein yield and size, strategies using synthetic genes designed with host-specific codons were developed. Additionally, completely novel silk proteins were created by hybridizing motifs from different silk types. Several groups have used these approaches to produce a variety of engineered silk proteins, most of which are based on the sequences of major and flagelliform spidroins [52–55]. Teulé and colleagues [56] developed an iterative polymerization strategy to produce very large and repetitive spider silk genes in a precisely controlled manner (Fig. 9.3). This technique is extremely powerful since it allows the construction of basic repeats that exactly reflect the native silk protein sequences or represent new engineered variants of native sequences by mixing and matching structural motifs in different ratios and combinations [57]. This approach is also extremely flexible

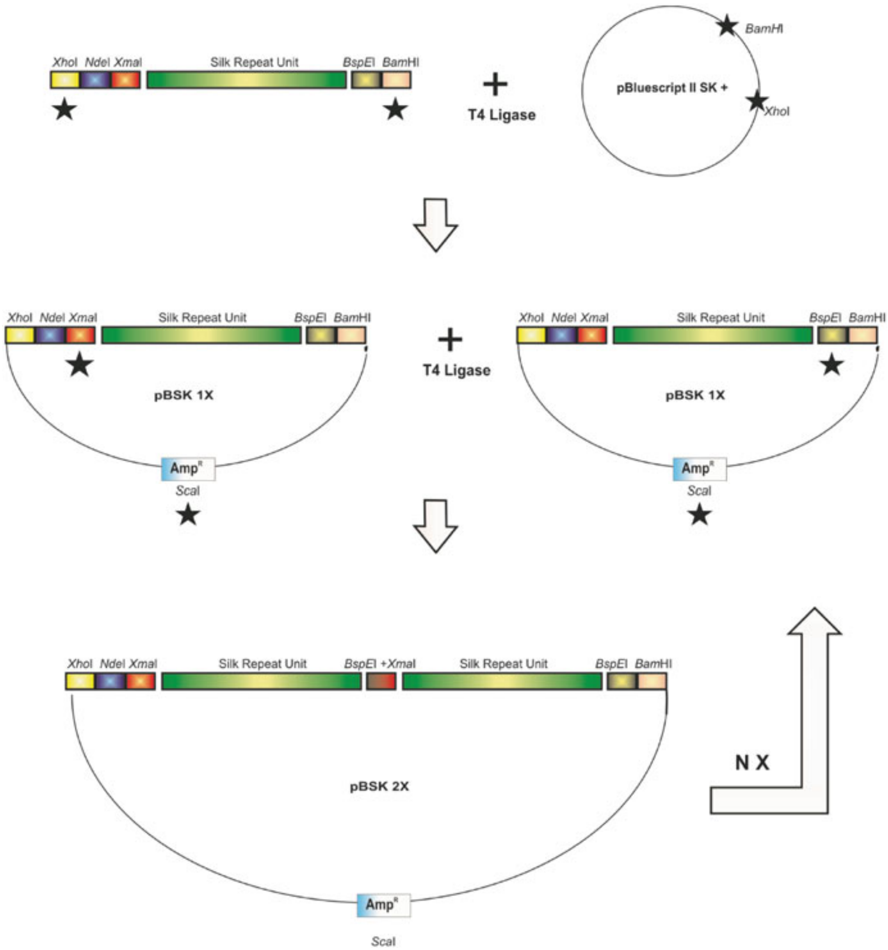


Fig. 9.3 Strategy for construction of a tandem spider silk synthetic gene based on a monomer with compatible but nonregenerable sites. ★ denotes DNA cut by a restriction enzyme. *N*× indicates that this strategy can be repeated as many times as one wishes (Reproduced with permission from Teulé et al. [56])

since two different recombinant plasmids containing silk multimer inserts at different stages of assembly can be used simultaneously as templates to generate a higher size multimer [56]. Utilizing a similar strategy, Xia et al [58] were able to produce a native-sized recombinant spider silk protein in a metabolically engineered *E. coli* with an elevated glycyl-tRNA pool. The 284.9 kDa recombinant protein was based on the MaSp1 motif from *N. clavipes*, which is extremely rich in glycine (43–45 %), and was produced by fed-batch culture reaching a protein yield of 2.7 g/L. In addition, the mechanical properties of the fibers spun with this ultrahigh molecular weight recombinant spider silk were comparable to those of the native one.

Thus, the high molecular weight of native spider silk proteins indeed plays an important role in determining its mechanical properties.

Another approach to producing recombinant spider silks utilizes transgenic animals and plants that carry a segment of silk DNA in their genome. Mice and goats have been transgenically modified to produce spider silk proteins in the female's mammary gland [59, 60]. However, the maximum protein yields observed for recombinant silk production in transgenic animals are low when compared with bacteria (117 mg/l and up to 300 mg/l, respectively) [19, 61, 62]. Scheller et al. [63] reported the expression of spider silk proteins in tobacco and potatoes for the first time. Using the *Cauliflower mosaic virus* (CaMV) 35S promoter, stable transgenic tobacco and potato lines were engineered to express different sizes of the *MaSp1* gene derivatives from *N. clavipes* in the endoplasmic reticulum (ER) of leaves. This technique has proven to be effective for the expression of MaSp1 and MaSp2 derivatives in greenhouse and field trials [23]. Additionally, recombinant MaSp1-like proteins were also produced in *Arabidopsis* leaves and seeds, as well as in somatic soybean embryos [22]. These results demonstrated that recombinant spider silk proteins had higher accumulation levels in seeds than in the leaves. Recently, the production of synthetic, high molecular weight spider silk proteins larger than 250 kDa based on the assembly of Flag protein monomers via intein-mediated trans-splicing was achieved in plants [64]. This method avoids the need for highly repetitive transgenes, which may result in higher genetic and transcriptional stability. The resulting multimeric structures formed microfibers with up to 500 μm in length and diameters ranging from 1 to 2 μm, thereby demonstrating their great potential as a biomaterial.

Because of their high capacity to produce natural silk, silkworms (*B. mori*) have also been studied as a host for the production of recombinant spider silk proteins. In a recent publication [65], transgenic silkworms were developed using a *piggyBac* vector composed of the *B. mori* fibroin heavy chain promoter and enhancer, a genetic sequence encoding a 78 kDa synthetic spider silk, and an enhanced green fluorescent protein (EGFP) tag (Fig. 9.4). The chimeric silkworm/spider silk fibers produced by the transgenic *B. mori* were significantly tougher than silkworm silk and as tough as dragline spider silk native fibers. These results showed that silkworms can be engineered to manufacture composite fibers containing stably integrated spider silk protein sequences with improved mechanical properties.

9.3.2 New Biomaterials from Recombinant Spider Silk Proteins

Silk proteins have been shown to solubilize in water, organic solvents, and ionic liquids, indicating the versatile options available [66]. In addition, depending on the processing route, phase transition of spider silk proteins is controlled by parameters of chemical and physical processes leading to different material morphologies,

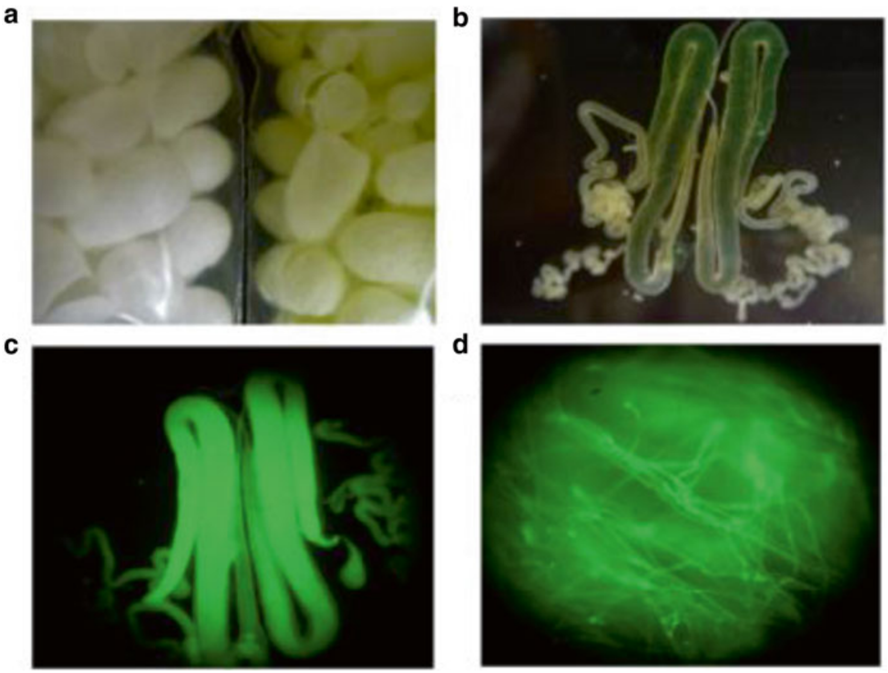


Fig. 9.4 Expression of a chimeric silkworm/spider silk/EGFP protein in *Bombyx mori* (**a**) cocoons, (**b**, **c**) silk glands, and (**d**) silk fibers (Reproduced with permission from Teulé et al. [65])

including fibers, films, gels, porous sponges, and other related systems (Fig. 9.5). For instance, a film can be formed by simple evaporation of the solvent, and if porogens are introduced into the silk protein solution prior to evaporation of the solvent, porous structures or foams can be produced [46].

Transparent films, ranging in thickness from 0.5 to 1.5 μm, have been produced using a hexafluoroisopropanol (HFIP) solution containing recombinant dragline silk proteins. Treating the films with potassium phosphate or methanol could convert the proteins' secondary structure from α-helix to β-sheet, making them water insoluble [67]. The portion of induced β-sheet structure can be regulated by the incubation time or by treatment of the film with methanol/water solutions mixed at different ratios. Different contents of β-sheet structure will not only determine the stability of silk films but will also influence their mechanical properties. Generally, a higher amount of β-sheet increases the elastic modulus and strength of the film but reduces its elasticity [46].

Microparticles are also one of the manifold morphologies spider silk proteins can adopt. These particles are formed by precipitating aqueous silk protein solutions with kosmotropic salts such as potassium phosphate or ammonium sulfate [68, 69]. The diameter of the spherical colloidal assemblies is variable (100 nm to 10 μm) and depends on the initial protein concentration and the chosen kosmotropic salt. For example, the addition of potassium phosphate (>300 mM) results in increased

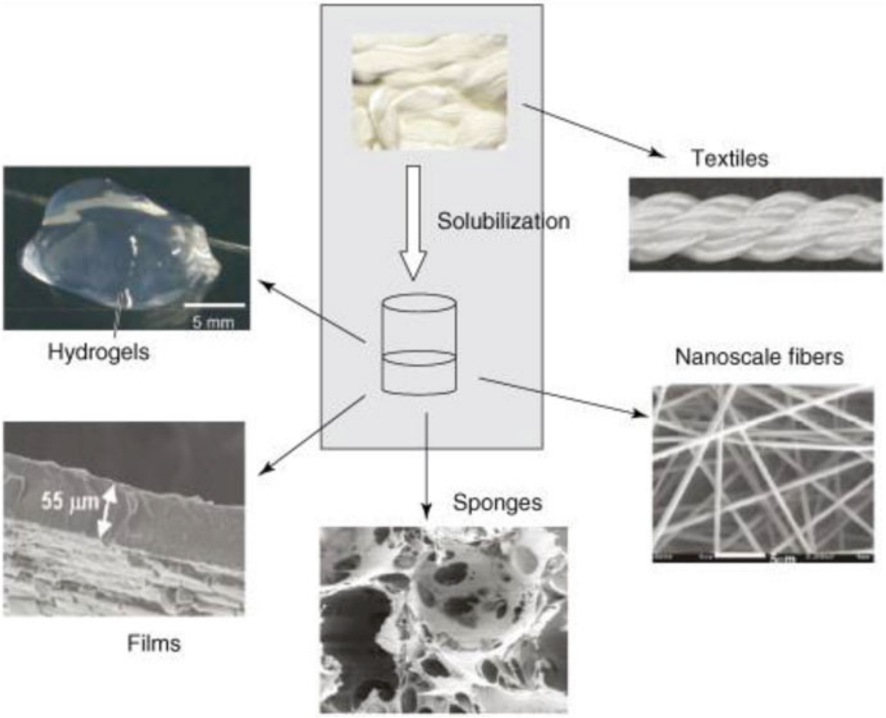
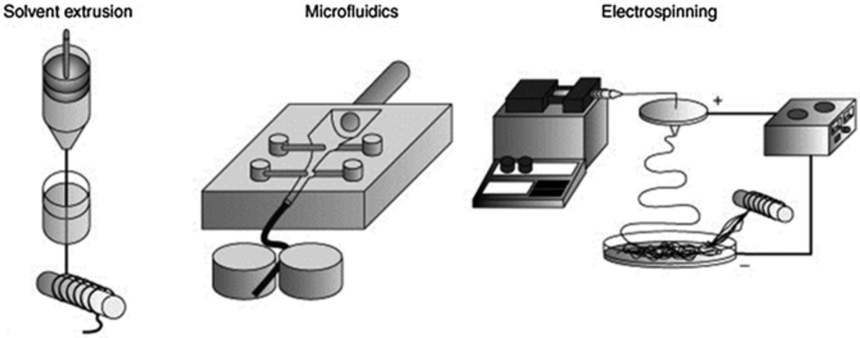


Fig. 9.5 Processing of silk into new biomaterials. Native and recombinant spider silks can be solubilized and processed into a range of different structures (Reproduced from Kluge et al. [66] with permission of Elsevier Limited)

hydrophobic interactions accompanied by a dense packing of the spider silk proteins. Such tight packing leads to the formation of chemically stable spheres, with a high content of β-sheet secondary structure and extremely smooth surfaces [70].

Despite the wide range of biomaterials that can be assembled from recombinant spider silk proteins, the mechanical properties exhibited by drawn synthetic fibers are still below the measured properties of native spider silks. Solvent extrusion [56], electrospinning [71], and microfluidics [72] are some of the techniques that have already been used to process recombinant spider silk proteins into fibers (Fig. 9.6). However, it is still difficult to manufacture long lengths of material in sufficient quantity and with superior quality. Moreover, most of the fibers created by spinning processes are so brittle that their mechanical properties cannot be properly measured [71, 73]. Even so, these studies are important to provide some understanding about the main keys to spinning recombinant silk proteins. For instance, drawing the newly formed fiber for strain elongation improves the mechanical properties of synthetic fibers by the induction of β-sheet formation [61, 74]. Future challenges will include the optimization of the spinning processes in order to scale up production and maximize the properties of the processed material.

a



b

	Solvent extrusion	Microfluidics	Electrospinning
Final fiber size	Micron-scale	Micron-nano scale	Nano-scale
Advantages	Well-established, inexpensive and simple	Highly biomimetic, multiple inputs, fine process control	Extremely fine diameters, simple operation
Disadvantages	Large-scale only	Not well-established, manufacturing challenges	Poor process control, poor functional properties, small-scale only

Fig. 9.6 Schematic representation of current silk fiber-forming techniques. (**a**) Solvent extrusion is performed by drawing the fiber through a coagulation bath in a controlled manner. Microfluidic devices use a contracting channel and multiple solvent inputs to regulate the geometry and chemistry of the resulting fiber. Electrospinning processes combine strong voltage gradients and syringe pump extrusion and result in either random or aligned fiber deposition. (**b**) Characteristics of the different fiber-forming processes. To date, electrospinning has proved to be the most useful approach, mainly because of the low amounts of spider silk materials needed and the utility of the resulting electrospun fiber mats for cell and tissue culture studies (Reproduced from Kluge et al. [66] with permission of Elsevier Limited)

9.3.3 Biotechnological Applications of Synthetic Spider Silk

Due to their outstanding mechanical properties, different biotechnological applications have been foreseen for spider silk proteins. They have desirable self-assembly characteristics, which allow their utilization as molecular building blocks for the production of different biomaterials. Furthermore, they can be genetically modified to allow the covalent coupling of peptides, enzymes, or particles before and after silk processing into biomaterials, therefore expanding the range of potential applications [75–78].

Recently, the synthesis of novel spider silk uranyl-binding proteins was demonstrated through bioengineering of varying repeats of a uranyl-binding sequence

adopted from a mutated calmodulin sequence in combination with spider silk sequences [79]. This new chimeric protein system can be used in environmental engineering as a substrate for uranium collection and removal. Once selective metal binding can be exploited, the development of such silk-based material might also find applications in microelectronics, biosensors, solar energy, and electrical batteries [79].

Spider silks also possess several desired characteristics for biomedical applications, such as biocompatibility, low inflammatory potential, antimicrobial activity, and a slow rate of degradation. The specific biomedical application of synthetic spider silks will not only rely on its molecular structure but also on the morphology adopted by the silk proteins. Silk fibroin films, for instance, can be used for different medical applications such as lenses, drug-loading films, and, mainly, cell-supporting scaffolds for tissue engineering [80–83]. Genes and drug delivery platforms can be developed using spider silk microcapsules [48, 84, 85]. Spider silk proteins can also be processed into coatings that can be used to improve the biocompatibility and surface properties of biomaterials, such as silicone implants [86].

Overall, the macroscopic structure of silk-based materials will be responsible for their interface with the environment, and the advantages and limitations of each system will determine its use in a specific application or for commercial exploitation [66].

9.4 Concluding Remarks

In the last few decades, several prokaryotic and eukaryotic hosts have been tested for the production of recombinant spider silk proteins. So far, there has been considerable progress in understanding the genetic organization of spider silk proteins and the self-assembly and processing of synthetic spider silks into many material formats. Recently, the production of native-sized (285 kDa) spider silk protein and the production of chimeric silkworm/spider silk fibers with improved mechanical properties have been reported [58, 65].

For any commercial application of synthetic spider silks, it is still necessary to develop new strategies for the production of homogeneous and reproducible fibers with defined mechanical properties. Moreover, applications of spider silks in commodity materials, such as textiles, high-performance composite materials, and durable and tough materials, in general, will require the success of robust and low-cost expression systems coupled with environmentally friendly assembly processes. Further exploitation of the aqueous processing and assembly of highly hydrophobic silk proteins into material structures together with a better comprehension of the natural events associated with the storage and spinning of the silk in spiders may be the keys to the development of a green improved biomaterial production and design.

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Chapter 10
Nano-Insecticides for the Control of Human and Crop Pests

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Abstract Nanotechnology is a promising field of interdisciplinary research. It opens up a wide array of opportunities in various fields like insecticides, pharmaceuticals, electronics, and agriculture. Biosynthesis of insecticides from plant extracts is currently under exploration. Plant extracts are very cost-effective and eco-friendly and thus can be an economic and efficient alternative for the large-scale synthesis of synthetic and other chemical insecticides. The present review was carried out to establish the management of insect pests using silver nanoparticles (AgNPs) from *Cassia occidentalis* against different life stages of crop and human pests. Synthesized AgNPs were characterized by UV–vis spectroscopy, scanning electron microscopy (SEM), energy-dispersive X-ray spectroscopy (EDX), and Fourier transform infrared spectroscopy (FTIR). Characteristics of the synthesized AgNPs were confirmed by analyzing the excitation of surface plasmon resonance (SPR) using a UV–vis spectrophotometer at 420 nm. SEM analysis of the synthesized AgNPs clearly showed clustered and irregular shapes, mostly aggregated and having a size of 20–85 nm. The chemical composition of elements present in the

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solution was determined by its energy-dispersive spectrum. The FTIR analysis of the nanoparticles indicated the presence of proteins, which may be acting as capping agents around the nanoparticles. Biosynthesis of nanoparticles may be triggered by several compounds such as carbonyl groups, terpenoids, phenolics, flavones, amines, amides, proteins, pigments, alkaloids, and other reducing agents present in the biological extracts. Overall, this study adds knowledge on *C. occidentalis-borne* insecticides and green-synthesized AgNP toxic against arthropods of medical and agricultural importance, allowing us to propose the tested products as effective candidates to develop newer and safer pest control tools.

Abbreviations

a.u.	Absorption unit
AgNP	Silver nanoparticle
CQ	Chloroquine
DMRT	Duncan's multiple range test
EDX	Energy-dispersive X-ray
fcc	Face-centered cubic
FTIR	Fourier transform infrared
IPM	Insect pest management
JCPDS	Joint Committee on Powder Diffraction Standards
KeV	Kiloelectronvolt
LC_{50}, LC_{90}	Lethal concentration
NPs	Nanoparticles
ppm	Parts per million
RBC	Red blood cell
SD	Standard deviation
SEM	Scanning electron microscopy/microscope
SPR	Surface plasmon resonance
UV–vis	UV–visible
WHO	World Health Organization
XRD	X-ray diffraction

10.1 Introduction

Arthropods are dangerous vectors of often deadly agents of diseases and parasites, which may hit as epidemics or pandemics in the increasing world population of humans and animals [1, 2]. Among them, mosquitoes (Diptera: Culicidae) represent a key threat for millions of people worldwide, since they act as vectors for important

pathogens, including malaria, dengue, yellow fever, Japanese encephalitis, West Nile, and parasites, such as filariasis. Malaria is caused by *Plasmodium* parasites vectored to vertebrates through the bites of infected *Anopheles* mosquitoes, which bite mainly between dusk and dawn. According to the latest estimates, there were about 198 million cases of malaria in 2013 and an estimated 584,000 deaths. Most deaths occur among children living in Africa, where a child dies every minute from malaria. Malaria mortality rates among children in Africa have been reduced by an estimated 58 % since 2000 [3, 4]. A great increase in the incidence of the malaria parasite, *P. falciparum*, resistant to commonly used drugs (e.g., chloroquine, CQ, hereafter), has occurred in a number of endemic areas and has been identified as one of the main factors responsible for high malaria-related mortality [4]. There is an urgent need to identify alternative drugs to treat malaria, particularly that caused by the drug-resistant *P. falciparum*. Since a number of currently employed drugs originated from medicinal plants (e.g., artemisinin and quinine), additional species reported by traditional medicine can be surveyed to identify novel compounds active against *Plasmodium* parasites [5–7].

Cassia occidentalis, a native plant of South India, commonly called coffee senna, belongs to the family Caesalpiniaceae. It is a pan-tropical plant species [8], used worldwide in traditional medicines [9]. The leaves are lanceolate or ovate-lanceolate; the leaflets, 3-paired, membranous, glaucous, ovate, or lanceolate; the flowers, yellow, in short racemes; the pods, recurved, glabrous, and compressed; and the seeds, dark olive green, ovoid, compressed, hard, smooth, and shining. *C. occidentalis* is used as a diuretic and in the treatment of snakebite [10]. Different parts of this plant have been reported to possess anti-inflammatory and antiplasmodial activities [11, 12]. A wide range of chemical constituents have been isolated from *C. occidentalis*, including sennoside, anthraquinone glycoside [13], fatty oils, flavonoid glycosides, galactomannan, polysaccharides, and tannins [14]. According to Haraguchi et al. [15, 16], anthraquinones were considered the secondary metabolites responsible for toxic effects, such as ataxia, muscle weakness, stubbing, body weight loss, and death induced by oral intake of *C. occidentalis* seeds in rats.

10.1.1 **Anopheles stephensi*: A Major Malaria Vector***

Anopheles stephensi Liston is the major human malarial mosquito vector prevalent in several countries including the Middle East and South Asia [17]. An obvious method for the control of mosquito-borne diseases is the use of insecticides, and many synthetic agents have been developed and employed in the field with considerable success. However, one major drawback with the use of chemical insecticides is that they are nonselective and can be harmful to other organisms in the environment. They also provoke undesirable effects, including toxicity to nontarget organisms, and raise environmental and human health concerns [18].

10.1.2 Plasmodium *Life Cycle*

The *Plasmodium* parasite is transmitted to humans by mosquitoes of the genus *Anopheles* when the insects bite and transfer saliva to the blood to prevent clotting. The parasites then infect hepatocytes where they rapidly multiply and eventually lyse their host cells. The ability of *P. ovale* and *P. vivax* to lie dormant in the hepatocytes for months to years, as indicated above, potentiates recrudescent disease at a later time. When released from infected hepatocytes, the parasites enter red blood cells (RBCs) and feed on hemoglobin. Eventually they reproduce to the point of lysing the RBCs, causing a new infective parasitemia and repeating the life cycle. Some parasites become gametocytes. When both male and female gametocytes are taken up by a biting mosquito, they reproduce in the insect, creating progeny ready to infect a new human host [19].

10.1.3 Spodoptera litura*: The Tobacco Cutworm*

Spodoptera species are among the most important insect pests of agricultural and horticultural crops. The cutworm, *S. litura* Fabricius (Lepidoptera: Noctuidae), is a polyphagous insect that has about 150 host species [20]. It is one of the most economically important insect pests of cotton, tobacco, chili, tomato, beetroot, cabbage, cauliflower, oil seeds, and other garden plants in many countries including India, Japan, China, and other countries of South East Asia and has been recorded as a cosmopolitan pest of sesame in Japan. *S. litura* is commonly known as the tobacco caterpillar, cotton leaf worm, chili fruit borer, and beet army worm. In recent years, *S. litura* has emerged as a major pest of groundnut in India, particularly in Andhra Pradesh [21]. This pest has acquired resistance to many conventional and currently available insecticides. Hence, there is a need to search for an alternate insecticide, which can fit into farmers' budgets as well into an integrated pest management program.

10.2 Nanotechnology

Nanotechnology is a promising field of interdisciplinary research, since it opens up a wide array of opportunities in different fields including pharmacology, electronics, parasitology, and pest management [22, 23]. Recently, it has been pointed out that the plant-mediated biosynthesis of nanoparticles is advantageous over chemical and physical methods because it is cheap and environment-friendly. Indeed, if compared to chemical, photochemical, and electrochemical reduction methods, as well as heat evaporation, the bio-reduction method does not require high pressure, energy, temperature, or the use of highly toxic chemicals [24–26]. A growing number

of plants and fungi have been proposed for efficient and rapid extracellular synthesis of silver and gold nanoparticles [27, 28], which show excellent mosquitocidal properties, including in field conditions [29–33]. In the present study, we propose a novel method of plant-mediated synthesis of silver nanoparticles using a plant leaf extract of *C. occidentalis* which acts as a reducing and capping agent (a capping agent is a strongly absorbed monolayer of usually organic molecules used to aid stabilization of nanoparticles). Silver nanoparticles were characterized by UV–visible (UV–vis) spectrum, Fourier transform infrared (FTIR) spectroscopy, energy-dispersive X-ray spectroscopy (EDX), and X-ray diffraction (XRD). The *C. occidentalis* extract and the green-synthesized silver nanoparticles were tested against the malaria vector *A. stephensi* and the crop pest *S. litura*.

10.3 Characterization of Nanoparticles

10.3.1 UV–Visible Absorption Spectroscopy Studies

Nanoparticles are primarily characterized by UV–vis spectroscopy, which proves to be a very useful technique for their analysis [34]. As a *C. occidentalis* leaf extract is mixed with an aqueous solution of silver nitrate, its color starts to change from yellowish to brown due to reduction of silver ion, which indicates the formation of silver nanoparticles (AgNPs) (Fig. 10.1a, b, c). It is generally recognized that

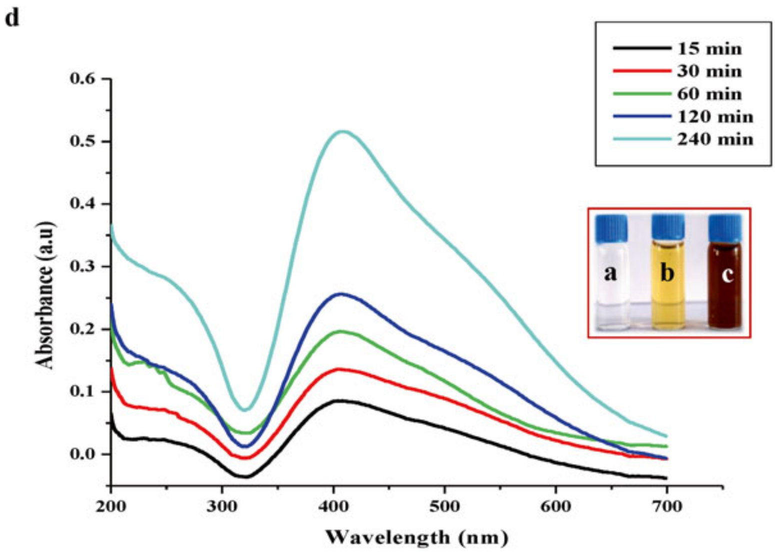


Fig. 10.1 Chromatic variation of the *Cassia occidentalis* leaf extract (**a**) before and (**b**) (**c**) after the process of reduction of Ag^+ to Ag nanoparticles. (**d**) UV visualization of the absorption spectra of *C. occidentalis*-synthesized Ag nanoparticles at different time intervals

UV–vis spectroscopy can be used to examine the size and shape of controlled nanoparticles in aqueous suspensions [35]. In our experiments, UV–vis spectra were recorded for the *C. occidentalis* leaf extract mixed with 1 mM $AgNO_3$ solution at various time intervals (15, 30, 60, 120, and 240 min).

The change of color at the different time periods may be due to a variation in concentration, size, and shape of the particles. Consequently, absorbance peaks can be used as a tool to predict particle size and stability. Smaller AgNPs will have an absorbance maximum around 420 nm, which increases with size and disappears when particle size falls outside nanodimensions. Earlier studies on the biosynthesis of gold nanoparticles using *P. aquilinum* extracts from the aerobic, gram-negative bacterium *Stenotrophomonas maltophilia*, as a living factory, suggested an absorption maximum at 530 nm [36], whereas we found a sharp shift in peak maxima at 420 nm which reached 0.5 a.u in 240 min (Fig. 10.1d). The surface plasmon peak of AgNPs at 420 nm increased steadily as the reaction time increased and the peak was saturated after 120 min, indicating that silver nitrate was completely reduced. The absorption peak also varied as the function of reaction time and the concentration of silver nitrate changed. Our results are similar to previous work where the color of fresh suspension of plant extract, *Vitex negundo*, and silver nitrate solution was also dark brown [37].

10.3.2 X-ray Diffraction (XRD) Studies

The phase formation of the synthesized AgNPs was analyzed employing X-ray diffraction which confirmed that the bioreduced metal nanoparticles were of elemental silver based on the existence of peaks [e.g., (111), (200), (220), (311)], and it matched with the standard Joint Committee on Powder Diffraction Standards (JCPDS). The XRD pattern of *C. occidentalis*-synthesized AgNP is reported in Fig. 10.2. We observed the presence of four high diffraction peaks at 38.44°, 44.05°, 64.75°, and 78.01°, indexing the Bragg's reflection planes (100), (103), (112), and (201), respectively (JCPDS) (file nos. 87–0598 and 41–1402). This confirmed the face-centered hexagonal structure of crystalline AgNP. Similarly, Rajeshkumar et al. [38] reported that the four distinct diffraction peaks of the 2θ values of 38.27°, 44.16°, 65.54°, and 77.42° could be assigned the plane of (1 1 1), (2 0 0), (2 2 0), and (3 1 1), respectively, indicating that the silver nanoparticles are face-centered cubic (fcc) and crystalline in nature. The synthesized silver nanoparticles are compared with standard silver nitrate and pure silver particles which are published by the JCPDS (file nos. 04–0783 and 84–0713). The XRD clearly shows that the four distinct planes for the silver nanoparticles synthesized from algal extract of *S. longifolium* are highly crystallized and purified.

The noise observed may be due to the presence of various crystalline biological macromolecules in the aqueous extract of *C. occidentalis*. The results also showed that the Ag^+ ions of silver nitrate were reduced to Ag^0, suggesting that the sharp Bragg peaks resulted from the capping agent's stabilizing of the nanoparticle.

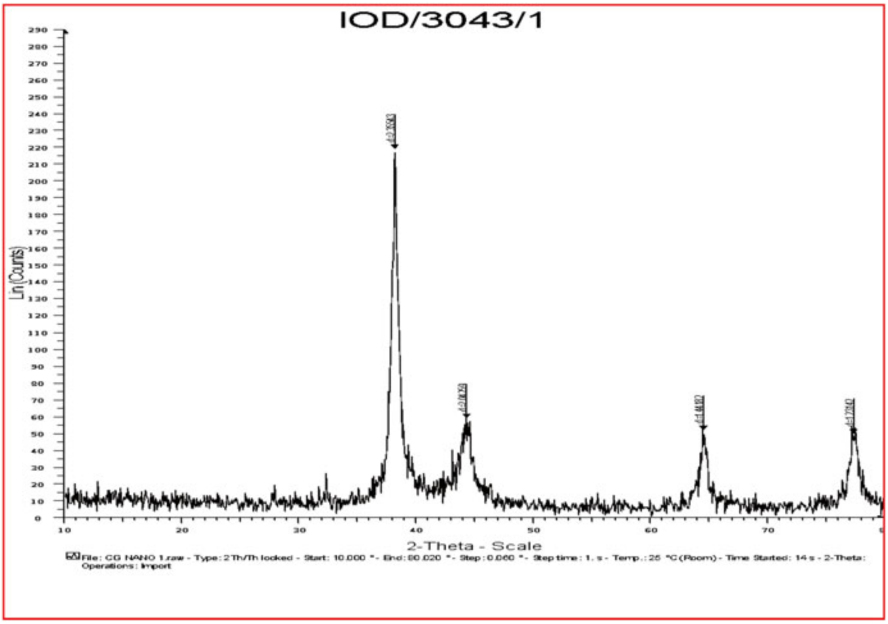


Fig. 10.2 X-ray diffraction pattern of synthesized silver nanoparticles using *Cassia occidentalis*

These results are in agreement with a previous study, where AgNPs were synthesized using a leaf extract of *Acalypha indica* to investigate their antibacterial activity against waterborne pathogens [39].

The XRD pattern of pure silver ions is known to display peaks at $2\theta = 7.9°$, 11.4°, 17.8°, 30.38°, and 44° [40]. XRD patterns of silver nitrate plus seaweed aqueous extract indicate that the structure of AgNPs is face-centered cubic [41, 32]. Hence, it is clear that AgNPs formed using *C. occidentalis* leaf broth were essentially crystalline. The XRD patterns displayed here are consistent with earlier reports [42]. Further, Dubey et al. [43] reported a size of 20–60 nm for silver nanocrystallites estimated from the full width at half maximum of the (111) peak of silver using the Scherrer formula. Therefore, the XRD results also suggest that crystallization of the bioorganic phase occurs on the surface of the AgNPs.

10.3.3 Fourier Transform Infrared Spectroscopy (FTIR) Studies

FTIR spectroscopy analysis was carried out to identify the biomolecules responsible for capping of the bioreduced AgNPs synthesized using plant extract. Figure 10.3 shows that the FTIR spectra of aqueous AgNPs prepared from the *C. occidentalis*

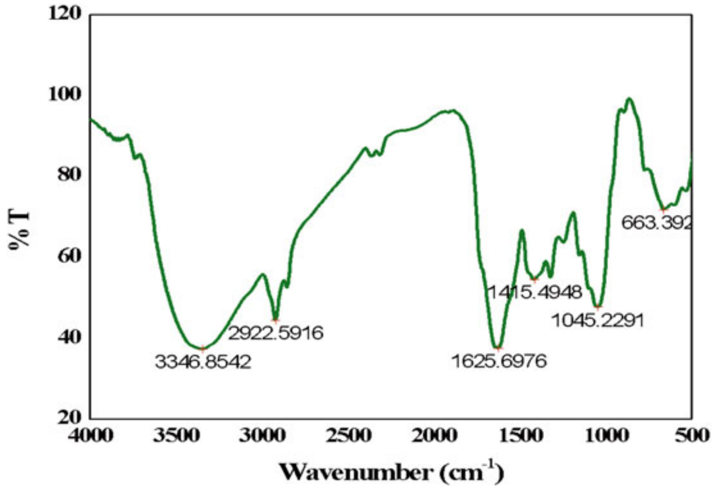


Fig. 10.3 FTIR spectra of silver nanoparticles synthesized with *Cassia occidentalis* leaf extract

leaf extract had peaks at 3346.85, 29.22.59, 1625.69, 1415.49, 1045.22, and 663 cm^{-1}. These results indicate the presence of various functional groups such as alkane groups, methylene groups, alkene groups, amine groups, and carboxylic acids. These functional groups are the major classes in many of the chemical groups that have proven potential as reducing agents in the synthesis of AgNPs [44].

The FTIR peak located at around 2,359 cm^{-1} is attributed to the N–H stretching vibrations or the C=O stretching vibrations. A broad intense band at 3,402 cm^{-1} in the spectra can be assigned to the N–H stretching frequency arising from the peptide linkages present in the proteins of the extract [45]. Thus, it appears conceivable that biological molecules from plants may perform dual functions of reduction and stabilization of AgNP in the aqueous medium, possibly by in situ oxidation of hydroxyl groups and by the intrinsic carbonyl groups, as well as those produced by oxidation with air [46, 32]. FTIR study reveals the multi-functionality of the *C. occidentalis* aqueous extract, where reduction and stabilization occur simultaneously. Figure 10.3 shows transmittance peaks at 612.28 (C–H bend alkenes), 1091.65 (C–N stretching vibration of aliphatic amines), 1440 (O–H bend carboxylic acids), 2923.56 (C–H stretch alkenes), and 3443.28 (O–H stretching alcohols group) in the FTIR spectra of aqueous AgNPs prepared from the *C. occidentalis* leaf extract, suggesting that these compounds may be responsible for production of AgNPs under these conditions. Such peaks indicate that the carbonyl group formed amino acid residues and that these residues capped the AgNPs to prevent agglomeration, thereby stabilizing the medium [47]. However, apparent contradictions have produced contention over AgNP toxicity and mode of action. AgNP size, size distribution, dispersion, and agglomeration state each appear to be drivers of AgNP toxicity, but the relative importance of each variable and their interaction is not fully understood [48]. FTIR peaks corresponding to aromatic rings, geminal methyls, and

ether linkages indicated the presence of flavones and terpenoids responsible for the stabilization of the AgNPs synthesized by a *Sesuvium portulacastrum* leaf extract [49]. Absorbance peaks at 1,620–1,636 cm^{-1} represent carbonyl groups from polyphenols such as catechin gallate, epicatechin gallate, epigallocatechin, epigallocatechin gallate, gallocatechin gallate, and theaflavin; these results suggest that molecules attached to AgNPs have free and bound amide groups. Amide groups may also be present in the aromatic rings. These observations lead to the conclusion that the compounds attached to AgNPs could be polyphenols with an aromatic ring and bound amide region [50].

10.3.4 Scanning Electron Microscope (SEM) and Energy-Dispersive X-Ray Analysis (EDX) Studies

For SEM studies, reaction mixtures were air-dried on silicon wafers, resulting in a "coffee-ring" phenomenon. It is well known that when liquids that contain fine particles are evaporated on a flat surface, the particles accumulate along the outer edge and form such typical structures [51]. Figure 10.4 shows SEM images obtained with 10 % *C. occidentalis* leaf broth at 95 °C. AgNPs synthesized under these conditions are spherical in shape with a size of 35–85 nm. In addition, the SEM images show that the capped silver particles are stable in solution for at least 8 weeks. Similarly, using SEM micrograph analysis, Ankanna et al. [52] reported that AgNPs produced

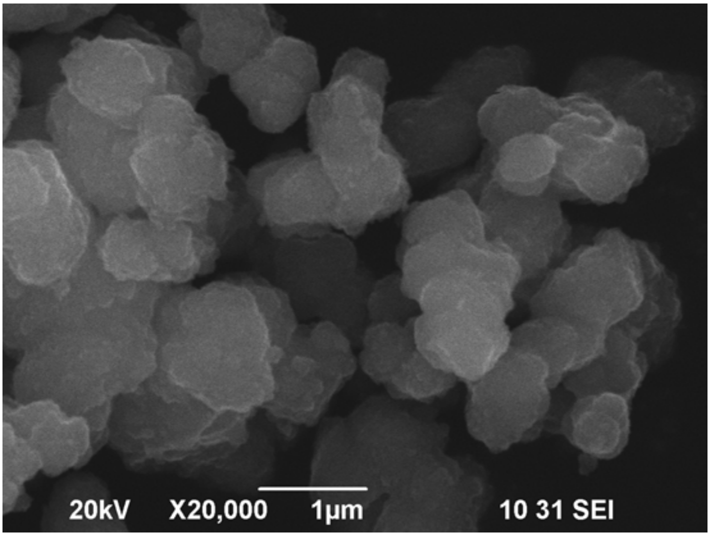


Fig. 10.4 Scanning electron micrograph of synthesized silver nanoparticles using *Cassia occidentalis* leaf broth

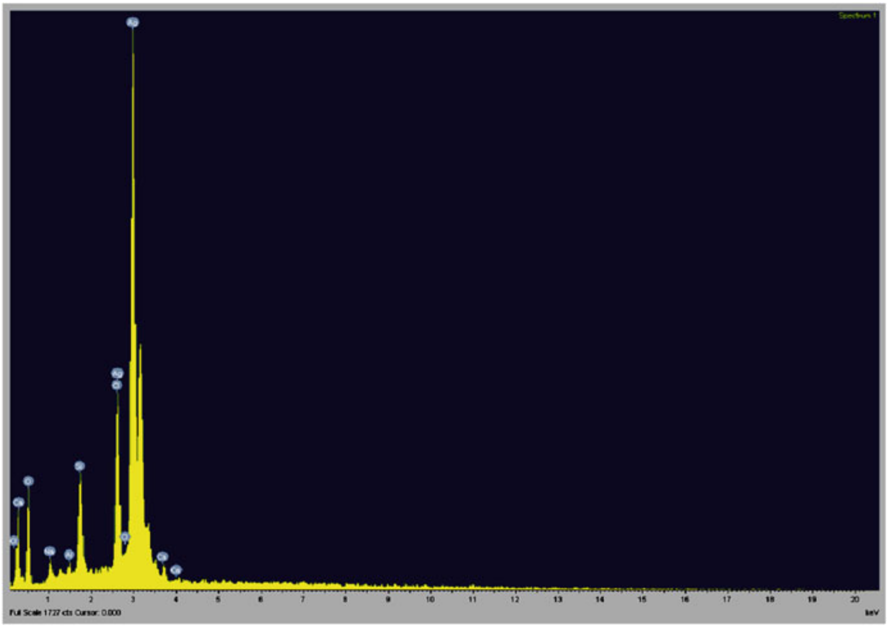


Fig. 10.5 EDX profile of synthesized silver nanoparticles using *Cassia occidentalis* leaf broth

with extracts of *Boswellia ovalifoliolata* were well dispersed and ranged in size 30–40 nm. The AgNPs formed under these conditions were predominantly cubical with uniform shape. It is known that the shape of metal nanoparticles considerably change their optical and electronic properties [53]. The particle shape of plant-mediated AgNPs are mostly spherical, with the exception of neem (*Azadirachta indica*), which yields polydispersed particles with both spherical and flat plate like morphology 5–35 nm in size [54].

Using SEM analysis, earlier authors reported fabrication of AgNPs ranging from 55 to 80 nm and triangular or spherical gold nanoparticles using a novel sundried biomass from *Cinnamomum camphora* leaf [46]. Spot EDX, which involves using an EDX attachment with an SEM, is known to provide information on the chemical analysis of the fields that are being investigated or the composition at specific locations. Figure 10.5 shows a representative profile of spot EDX analysis which reveals a strong signal in the silver region and confirms the formation of AgNPs. A distinct signal and high atomic percent values for silver were obtained. These results are consistent with an earlier report on AgNP synthesis using a filtrate from the fungus *Trichoderma viride* [55]. Metallic silver nanocrystals generally show a typical optical absorption peak at approximately 3 keV due to surface plasmon resonance [56]. A weak recorded signal from "O" may be due to the presence of organic moieties from the enzymes or proteins in the leaf extract. This interpretation is consistent with reports that nanoparticles synthesized using plant extract are surrounded by a thin layer of some capping organic material from the plant leaf broth, which remains stable in solution even after synthesis.

10.4 Experiments on Treatment of Two Model Insect Pests with *C. occidentalis* Extracts and AgNPs

In this section we report the relative effectiveness of treatment with ethanolic extracts or AgNPs produced from extracts of *C. occidentalis* on larval and pupal mortality and adult longevity and fecundity of *S. litura* and *A. stephensi*. AgNPs were consistently more effective in terms of concentrations required to impact these parameters. Experimental results are presented below.

The larval and pupal mortality of *S. litura* after the treatment of ethanolic extract of *C. occidentalis* are shown in Table 10.1. Considerable mortality was evident after treatment of ethanolic extract of *C. occidentalis* at different life stages. The LC_{50} values varied approximately 2.3-fold, from 216.31 ppm for first instar larvae to 494.48 ppm for the pupal stage; the overall variation in LC_{90} values was somewhat narrower, at 1.8-fold, from 541.35 ppm for first instar to 998.16 ppm for pupa.

The larvicidal and pupicidal toxicity of AgNPs are presented in Table 10.2. Graded concentrations (2, 4, 6, 8, and 10 ppm) of AgNPs were used to treat first to sixth instar larvae and pupae of *S. litura*. The LC_{50} values ranged from 3.80 ppm for first instar to 6.68 ppm for sixth instar or 1.8-fold; again, the LC_{90} values had a slightly narrower range, from 8.58 ppm for first instar to 13.31 ppm for sixth instar or approximately 1.6-fold. The LC_{50} value for pupae was comparable to the last larval stage, at 14.01 ppm, but the LC_{90} value for pupae was 26.54 ppm, nearly 2-fold higher than for the last instar larvae.

In acute toxicity experiments, the ethanolic extract of *C. occidentalis* was toxic against larval instars (I–IV) and pupae of *A. stephensi*, even if tested at low doses. LC_{50} values were 143.35 ppm (first instar), 167.81 ppm second instar, 201.64 ppm third instar, 250.13 ppm fourth instar, and 347.44 ppm (pupae) (Table 10.3). Toxicity results indicated that the percentage of mortality was proportional to the concentration of *C. occidentalis* extract.

The early instar larvae were more susceptible than the later ones and the pupae, which were not much affected by the *C. occidentalis* ethanolic extract. Plant compounds with larvicidal activity have been investigated for inhibitory effect on acetylcholinesterase and detoxifying enzymes of insects aiming to identify agents that can be used as synergists for insecticides routinely used in control programs [57, 58].

These results are comparable to earlier reports of Prabhu et al. [59] who reported that methanolic extract of *M. oleifera* exhibited dose-dependent larval toxicity against *A. stephensi*. LC_{50} values were 57.79 ppm first instar, 63.90 ppm second instar, 72.45 ppm third instar, 78.93 ppm fourth instar, and 67.77 ppm (pupae).

Murugan and Jeyabalan [60] reported that *Leucas aspera*, *Ocimum sanctum*, *A. indica*, *Allium sativum*, and *Curcuma longa* had a strong larvicidal, anti-emergence, adult repellency, and anti-reproductive activity for *A. stephensi*. Rawani et al. [61] established the larvicidal properties of crude extracts of *Carica papaya*, *Murraya paniculata*, and *Cleistanthus collinus* against the mosquito, *Culex quinquefasciatus*, and suggested that the presence of many bioactive principles such as steroids, alkaloids, terpenes, and saponins may be responsible for their biocontrol potential.

Table 10.1 Larvicidal and pupicidal effect of *Cassia occidentalis* leaf ethanolic extract on *Spodoptera litura*

	% of larval and pupal mortality (mean ± SD)						95 % confidence limit		
	Concentration (ppm)								x^2
Life stages	100	200	350	500	650	LC_{50} (LC_{90}) (ppm)	LC_{50} (*LFL–UFL*) (ppm)	LC_{90} (*LFL–UFL*) (ppm)	
first instar	35.4 ± 1.32^{c}	49.1 ± 1.64^{d}	64.6 ± 2.02^{e}	82.4 ± 2.25^{e}	100 ± 0.0^{a}	216.31 (541.35)	74.49-302.59	431.33-820.79	8.26*
second instar	32.9 ± 1.42^{cd}	45.2 ± 1.38^{c}	59.5 ± 1.17^{c}	79.8 ± 1.62^{d}	100 ± 0.0^{a}	240.95 (565.04)	83.85-339.60	441.51-928.34	10.67*
third instar	30.1 ± 1.91^{d}	41.3 ± 1.73^{d}	52.4 ± 1.12^{c}	71.2 ± 2.10^{e}	94.3 ± 1.52^{cd}	283.50 (669.03)	164.72-373.99	536.61-1004.32	6.84*
fourth instar	28.1 ± 1.33^{c}	37.4 ± 1.49^{cd}	48.7 ± 1.81^{d}	65.4 ± 1.98^{de}	89.5 ± 1.55^{cd}	319.49 (742.42)	278.50-358.30	665.64-854.56	5.04*
fifth instar	26.2 ± 1.15^{c}	34.6 ± 1.37^{c}	41.9 ± 1.65^{d}	62.4 ± 1.25^{c}	86.2 ± 1.72^{d}	354.31 (792.58)	251.85-457.79	631.60-1212.99	5.86*
sixth instar	21 ± 1.58^{d}	29.7 ± 1.24^{c}	36.9 ± 1.91^{de}	52.8 ± 1.10^{c}	74.9 ± 1.05^{bc}	435.62 (938.69)	389.95-489.04	822.80-1121.26	2.59*
Pupae	12.4 ± 1.02^{bc}	27.3 ± 0.98^{b}	35.9 ± 1.22^{c}	49.7 ± 1.44^{cd}	64.8 ± 1.30^{c}	494.48 (998.16)	446.00-556.53	872.38-1198.48	1.95*

Within a column means followed by the larval mortalities are expressed as mean ± SD of five replicates. Nil mortality was observed in the control. Within column means followed by the same letter(s) are not significantly different at the 5 % level by Duncan's multiple range test

LFL lower fiducial limit, *UFL* upper fiducial limit, x^2 chi-square value

*Significant at $P < 0.05$ level

Table 10.2 Larvicidal and pupicidal effect of *Cassia occidentalis*-synthesized AgNp treatment of *Spodoptera litura*

Life stages	% of larval and pupal mortality (mean ± SD) Concentration (ppm)					LC_{50} (LC_{90}) (ppm)	95 % confidence limit		x^2
	2	4	6	8	10		LC_{50} (*LFL–UFL*) (ppm)	LC_{90} (*LFL–UFL*) (ppm)	
first instar	37.3 ± 1.52^{cd}	51.7 ± 1.25^{c}	61.6 ± 1.74^{d}	85.8 ± 1.45^{cd}	100 ± 0.0^{a}	3.80 (8.58)	0.55-5.37	6.73-14.90	12.38*
second instar	34.2 ± 1.38^{c}	47.4 ± 1.83^{d}	57.2 ± 1.62^{d}	81.6 ± 2.09^{e}	100 ± 0.0^{a}	4.21 (9.02)	0.73-5.96	6.98-17.23	14.87*
third instar	31.6 ± 1.25^{c}	44.2 ± 1.53^{cd}	54 ± 1.98^{de}	78.9 ± 1.72^{d}	100 ± 0.0^{a}	4.51 (9.27)	1.01-6.42	7.12-18.81	16.73*
fourth instar	29.2 ± 1.37^{c}	39.8 ± 1.48^{cd}	50.9 ± 1.65^{d}	74.3 ± 1.85^{d}	97.5 ± 1.72^{d}	4.91 (9.91)	2.27-6.70	7.76-17.90	13.54*
fifth instar	24.7 ± 1.17^{c}	35.7 ± 1.22^{c}	44.2 ± 2.45^{e}	65.9 ± 1.54^{cd}	89.4 ± 1.99^{de}	5.70 (11.42)	4.08-7.21	9.25-17.41	7.45*
sixth instar	19.8 ± 1.22^{c}	32.5 ± 1.84^{d}	39.2 ± 1.46^{c}	56.7 ± 1.72^{d}	78.3 ± 2.05^{de}	6.68 (13.31)	4.08-7.21	11.90-15.47	3.11*
Pupae	11.8 ± 1.83^{d}	15.3 ± 1.10^{c}	19.2 ± 1.55^{d}	25.9 ± 1.42^{c}	35.6 ± 1.30^{c}	14.01 (26.54)	11.48-20.19	20.31-42.49	0.35*

Within a column means followed by the larval mortalities are expressed as mean ± SD of five replicates. Nil mortality was observed in the control. Within column means followed by the same letter(s) are not significantly different at the 5 % level by Duncan's multiple range test

LFL lower fiducial limit, *UFL* upper fiducial limit, x^2 chi-square value

*Significant at $P < 0.05$ level

Table 10.3 Larvicidal and pupicidal effect of *Cassia occidentalis* ethanolic extract on the malarial vector *Anopheles stephensi*

Life stages	% of larval and pupal mortality (mean ± SD) Concentration (ppm) 50	150	250	350	450	LC_{50} (LC_{90}) (ppm)	95 % confidence limit LC_{50}(*LFL–UFL*) (ppm)	LC_{90} (*LFL–UFL*) (ppm)	x^2
first instar	37.6 ± 1.35^{c}	48.6 ± 1.49^{cd}	64.6 ± 2.05^{de}	82.4 ± 1.01^{bc}	98.1 ± 1.03^{bc}	143.35 (400.04)	39.22-205.13	319.52-595.29	7.00^{*}
second instar	33.5 ± 1.08^{c}	44 ± 2.16^{e}	61.3 ± 1.24^{c}	77.4 ± 1.42^{c}	95.0 ± 0.81^{b}	167.81 (439.48)	137.03-194.12	396.84-499.46	4.62^{*}
third instar	28.1 ± 2.45^{e}	40.3 ± 1.24^{c}	57.3 ± 1.69^{d}	71 ± 0.81^{b}	87 ± 2.16^{de}	201.64 (509.38)	170.05-229.89	455.68-588.09	0.79^{*}
fourth instar	24.3 ± 1.69^{cd}	33.6 ± 1.24^{c}	53.4 ± 1.11^{b}	63.6 ± 2.05^{e}	75.1 ± 0.65^{b}	250.13 (613.40)	216.61-283.63	537.74-732.51	0.80^{*}
Pupae	21 ± 0.81^{b}	28.3 ± 1.24^{c}	41 ± 2.16^{e}	51.6 ± 1.69^{d}	59.6 ± 0.47^{a}	347.44 (820.31)	303.35-409.25	684.64-1069.99	0.31^{*}

Within a column means followed by the larval mortalities are expressed as mean ± SD of five replicates. Nil mortality was observed in the control. Within column means followed by the same letter(s) are not significantly different at the 5 % level by Duncan's multiple range test

LFL lower fiducial limit, *UFL* upper fiducial limit, x^2 chi-square value

*Significant at $P < 0.05$ level

C. occidentalis-synthesized AgNPs were highly effective in laboratory experiments conducted against *A. stephensi* larvae and pupae. LC_{50} values were 4.97 ppm first instar, 5.85 ppm second instar, 7.60 ppm third instar, 9.65 ppm fourth instar, and 14.15 ppm (pupae) (Table 10.4). Similarly, larvicidal activity of synthesized AgNPs using *Eclipta prostrata* was observed against *C. quinquefasciatus*. The LC_{50} and LC_{90} values were $LC_{50}=27.49$ and 4.56 mg/l and $LC_{90}=70.38$ and 13.14 mg/l and against *A. subpictus* $LC_{50}=27.85$ and 5.14 mg/l and $LC_{90}=71.45$ and 25.68 mg/l [62]. Further, Soni and Prakash [63] reported comparable results studying *Chrysosporium tropicum*-synthesized AgNPs tested against third instar larvae of *A. aegypti*, with an LC_{50} of 4 ppm, LC_{90} of 8.91 ppm, and an LC_{99} of 13.18 ppm.

C. occidentalis-synthesized AgNPs were highly toxic against *A. stephensi* larvae and pupae, even when tested at very low doses. Here mortality increased as the concentration increased, for example, in the first instar stage at 3.125 ppm, the larval mortality was 37.3 %, whereas at 50 ppm, it increased to 100 %. Pupal mortality at 3.125 ppm was only 14 %, but it increased to 66.3 % at 50 ppm (Table 10.4).

The larvicidal properties of AgNPs may be accounted for by their effect on structural deformation of DNA and digestive tract enzymes and generation of reactive oxygen species [64]. An increased larvicidal spectrum may also be due to the synergistic combination of AgNPs and proteins and other secondary metabolites adhering to the surface of AgNPs during their reduction and stabilization.

The experimental results from use of AgNPs were assessed to be more toxic to immature stages of *A. stephensi*; that is, the early instar larvae were much more susceptible than the later ones. The strong inhibitory effect of AgNPs on mosquito larvae may be due to their easy penetration. Smaller particles may easily enter the cuticle and body cells and arrest molting and other biochemical activities, which leads to the death of the insect. The reported mode of action for insecticidal activity of nanosilica is through desiccation of insect cuticle by physisorption of lipid which is also expected to cause damage to the cell membrane, again resulting in cell lysis and death [65]. Results from a recent study by Priyadarshini et al. [66] suggest that synthesized silver nanoparticles are a rapid, eco-friendly, and single-step approach; the AgNPs formed can be potential mosquito larvicidal agents.

Results showed that adult longevity and fecundity of *A. stephensi* were significantly reduced after the treatment with ethanolic extract of *C. occidentalis* and *C. occidentalis*-synthesized AgNP (Table 10.5). Longevity was reduced to 9 days after treatment at 500 ppm concentration of aqueous extract of *C. occidentalis*, whereas the control longevity was 20 days. Female fecundity was also highly reduced after the treatment with *C. occidentalis* extract and *C. occidentalis*-synthesized AgNP; 140 eggs were recorded in control while 127, 104, 87.3, 68.6, and 55.9 eggs were recorded in treated insects at 2, 4, 6, 8, and 10 ppm, respectively (Table 10.5).

In this study, AgNPs proved to have strong effects on longevity and fecundity in *A. stephensi*, which showed low concentrations in these parameters in comparison with ethanolic extracts of *C. occidentalis*. Higher concentrations of AgNPs greatly affected longevity, whereby the timing of adult emergence was very short and emerged adults died within a day or two. Entry into the pupal stage was much earlier after treatment, and some insects failed to pupate and some failed to emerge.

Table 10.4 Larvicidal and pupicidal effect of *Cassia occidentalis*-synthesized AgNPs on the malarial vector *Anopheles stephensi*

Life stages	% larval and pupal mortality (mean ± SD) Concentration (ppm)					LC_{50} (LC_{90}) (ppm)	95 % confidence limit		x^2
	2.5	5	10	20	40		LC_{50}(*LFL–UFL*) (ppm)	LC_{90} (*LFL–UFL*) (ppm)	
first instar	41.3 ± 0.47^{a}	52 ± 0.73^{b}	66.3 ± 1.24^{c}	82.5 ± 1.47^{cd}	100 ± 0.0^{de}	4.97 (23.00)	2.60-6.85	19.85-27.84	2.00^{*}
second instar	39.1 ± 0.98^{bc}	50 ± 0.81^{b}	63.4 ± 1.38^{c}	80.3 ± 1.24^{c}	100 ± 0.0^{de}	5.85 (24.07)	3.62-7.70	20.85-28.98	2.33^{*}
third instar	35.3 ± 2.65^{e}	45.6 ± 2.05^{de}	58 ± 0.81^{b}	75.6 ± 0.47^{a}	93.7 ± 1.95^{de}	7.60 (32.89)	4.78-10.01	28.50-39.45	1.83^{*}
fourth instar	32.8 ± 1.05^{bc}	42 ± 1.41^{cd}	54.3 ± 1.88^{d}	71.4 ± 1.22^{c}	88 ± 2.16^{e}	9.65 (39.61)	6.53-12.39	34.06-48.14	3.12^{*}
Pupae	29.6 ± 0.47^{a}	37 ± 0.89^{b}	$50.2 \pm .0.70^{b}$	64.3 ± 0.90^{bc}	75 ± 0.97^{bc}	14.15 (55.36)	2.85-24.63	38.18-129.01	5.96^{*}

Within a column means followed by the larval mortalities are expressed as mean ± SD of five replicates. Nil mortality was observed in the control. Within column means followed by the same letter(s) are not significantly different at the 5 % level by Duncan's multiple range test

LFL lower fiducial limit, *UFL* upper fiducial limit, x^2 chi-square value

*Significant at $P < 0.05$ level

Table 10.5 Fecundity and longevity of malarial vector, *Anopheles stephensi*, after treatment with *Cassia occidentalis* ethanolic extracts and AgNPs

Treatment (ppm)		Adult longevity (days) Male	Adult longevity (days) Female	Fecundity
Control		20 ± 0.1^{a}	35 ± 0.9^{b}	140 ± 0.6^{b}
C. occidentalis ethanolic extract	100	18.9 ± 1.2^{c}	33.3 ± 1.7^{d}	130 ± 1.2^{c}
	200	17.3 ± 1.4^{cd}	31.4 ± 0.5^{b}	110 ± 0.9^{bc}
	300	15.4 ± 0.8^{b}	27.5 ± 1.7^{d}	98.4 ± 2.5^{ef}
	400	11.4 ± 1.6^{d}	24 ± 1.2^{c}	85 ± 0.6^{b}
	500	9 ± 0.2^{a}	19.7 ± 1.3^{c}	62 ± 1.4^{cd}
AgNPs	2	17.6 ± 1.0^{b}	32.1 ± 1.5^{cd}	127 ± 1.7^{d}
	4	15.8 ± 1.4^{c}	29.5 ± 0.6^{b}	104 ± 0.5^{a}
	6	12.3 ± 0.4^{a}	25.6 ± 1.5^{cd}	87.3 ± 1.2^{c}
	8	10.5 ± 1.6^{d}	22 ± 1.4^{c}	68.6 ± 0.5^{a}
	10	8 ± 0.1^{a}	17.6 ± 1.5^{d}	55.9 ± 1.3^{c}

Means ± standard deviation (SD) followed by the same letter within rows indicates no significant difference (Duncan's multiple range test, $P < 0.05$)

Table 10.6 Adult longevity after the treatment of *Spodoptera litura* with *Cassia occidentalis* ethanolic extracts and AgNPs

Treatment (ppm)		Adult longevity (Days) Male	Adult longevity (Days) Female
Control		8.2 ± 1.52^{e}	7.2 ± 1.20^{d}
C. occidentalis ethanolic extract	100	7.6 ± 1.12^{de}	6.5 ± 1.01^{c}
	200	6.8 ± 0.98^{e}	5.7 ± 0.69^{d}
	300	5.6 ± 1.05^{d}	4.8 ± 0.92^{e}
	400	4.5 ± 1.27^{c}	3.9 ± 0.55^{e}
	500	3.8 ± 0.76^{bc}	3.0 ± 0.41^{b}
AgNPs	20	7.1 ± 1.02^{c}	6.2 ± 0.64^{d}
	40	5.9 ± 0.67^{d}	5.1 ± 0.12^{e}
	60	4.8 ± 0.54^{de}	4.0 ± 0.22^{d}
	80	3.6 ± 0.76^{d}	3.2 ± 0.45^{c}
	100	2.6 ± 0.41^{c}	2.0 ± 0.58^{b}

Means ± standard deviation (SD) followed by the same letter within rows indicates no significant difference (Duncan's multiple range test, $P < 0.05$)

Additionally, the emerged adults showed significant physiological abnormalities, with defective wings, legs, and abdomens. In agreement with our results, Kumar et al. [67] have reported a reduction in *A. stephensi* adult longevity (4.2 days in male and 11.7 days in female at 10 ppm) after the treatment with AgNP synthesized using *Annona squamosa*.

Treatments with *C. occidentalis* ethanolic extract and *C. occidentalis-synthesized* AgNP negatively affected adult longevity and fecundity in the cutworm *S. litura* (Table 10.6). Longevity was reduced to 3.8 days after treatment with 500 ppm

Table 10.7 In vitro antiplasmodial activity of the ethanolic extract of *Cassia occidentalis* against *Plasmodium falciparum* 3D7

	IC_{50} Values (%)	
Concentration (μg/ml)	Parasites counted	% of inhibition
25	61.8 ± 1.2^{e}	30.1^{e}
50	42.5 ± 1.6^{d}	51.9^{d}
75	31.2 ± 1.0^{c}	64.7^{c}
100	18.6 ± 0.6^{b}	78.9^{b}
Positive control, chloroquine	1.0 ± 0.0^{a}	100^{a}
Negative control	88.5 ± 1.7	0

Means ± standard deviation (SD) followed by the same letter within rows indicates no significant difference (Duncan's multiple range test, $P < 0.05$)

concentration of *C. occidentalis* ethanolic extract, while the control was 8.2 days (Table 10.6). To the best of our knowledge, this is the first study evaluating the impact of *C. occidentalis*-synthesized nanoparticles on longevity and fecundity of moth pests. More evidence is available on the toxicity of plant extracts alone against crop pests.

In this study, Table 10.7 shows the in vitro antiplasmodial activity of ethanolic leaf extract of *C. occidentalis* against *P. falciparum* 3D7, a CQ-sensitive strain. Using concentrations in μg/ml, 25 μg/ml showed a moderate activity of 30.1 % inhibition with a growth rate of 61.8 ± 1.2 %; a higher concentration such as 100 μg/ml showed a rapid reduction in growth rate to 18.6 ± 0.6 % with an inhibition of 78.9 %. The inhibition calculated from the parasite growth versus the negative control was 88.5 ± 1.7 %, whereas there was no growth in the positive control (CQ), which had an inhibitory effect of 100 %. In the present study, comparison of the extract-treated culture with a CQ treated culture showed a monotonic effect. These results are comparable to earlier findings of Gessler et al. [68] who conducted an in vitro study of crude ethanolic, petroleum ether, ethyl acetate, and water fractions of *Achyranthes aspera*, which showed antimalarial activity with IC_{50} median values of 78, 72, 3.0, and >500 μg/mL, respectively, against *P. falciparum* (KI). These results are comparable to earlier findings of Ponarulselvam et al. [69], who studied antiplasmodial activity of synthesized AgNPs using leaf extracts of *Catharanthus roseus*, yielding an (IC_{50}) value of 20 ± 0.7 % at 25 μg/ml. Panneerselvam et al. [70] also studied the potential antiplasmodial activity of synthesized AgNPs using *Andrographis paniculata*, which gave IC_{50} values of 26 ± 0.2 % with 25 μg/ml and 83 ± 0.5 % with 100 μg/ml. Further, Ravikumar et al. [71] reported the antiplasmodial potential of *C. roseus*, *Coccinia grandis*, *Thevetia peruviana*, *Prosopis juliflora*, *Acacia nilotica*, *A. indica*, and *Morinda pubescens* against *P. falciparum*. For instance, the ethanol leaf extract of *S. occidentalis* from Congo showed a higher in vitro antimalarial activity against *P. falciparum* CQ-sensitive strains, with an $IC_{50} < 3$ μg/mL [12].

10.5 Conclusion

In conclusion, data presented here using green synthesis shows that the environmentally benign and renewable source of *C. occidentalis* can be used as an effective reducing agent for the synthesis of AgNPs. The AgNPs formed in this way demonstrated significant larvicidal and pupicidal activity against both human and crop pests. Further studies on the isolation of active compounds from *C. occidentalis* plants for larval control and commercial preparation of repellent products and field trials are needed for effective bio-management of these and other insect pests.

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